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REPORT ON THE USE OF THE PHOTRONREFLECTOMETER IN SEROLOGICAL COMPARISONS

(Seven figures)

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THERE is a distinct need for an accurate and *rapid* method for the quantitative measurement of the intensity of precipitin reactions. The measurement of the volumes of precipitate or the determination of the nitrogen contents of washed precipitates are too laborious to be ideal methods for extensive use in serological comparisons. The development of the "photronreflectometer" by Raymond Libby seems to offer a method combining accuracy and ease in titrations, and it is our purpose to present typical results so far obtained with this instrument, together with a brief discussion of their implications.

MATERIALS AND METHODS

THE PHOTRONREFLECTOMETER

The photronreflectometer is a photoelectric instrument for the measurement of turbidity. The essentials of its mechanism have been described by its designer, Raymond Libby (1938*a*). Light from a constant source passes through the cell containing the turbid system to be measured. The galvanometric readings correspond to the deflection of light from the particles in suspension in the turbid system (Libby, 1938*a*, *b*). What is further required regarding the technique involved is a statement in detail describing the particular dilutions of antigen and antibody used in our procedure, together with an explanation of the manner of mixing the reagents, preparing the controls, incubating, recording the results, and interpreting the data.

THE PREPARATION OF ANTIGENS AND ANTISERA

The technique used by us has been briefly described (Boyden, 1942). Each precipitating antiserum is usually prepared in a common manner by injecting a rabbit intravenously with doubling doses of antigen on alternate days for four injections, the first dose containing 5 mg. protein per kilogram body weight of the injected rabbit. The rabbit is bled completely by cardiac puncture 7–10 days after the last injection and the blood allowed to clot. The antiserum, obtained after centrifuging, is sterilized

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by filtration through Seitz filters and stored in small vials in the icebox.

The antigens have been variously prepared, depending on the particular phases of the general problem of animal relationship being investigated. Generally, fresh sera are sterilized by Seitz filtration and stored in the icebox. In some cases the antigens have been obtained in the field and preserved with ether-phenol or merthiolate before filtration. In other experiments purified antigens, and in still others antigens extracted with petroleum-ether, were used. The details are given below in connection with each of the phases of this investigation.

THE TYPICAL TITRATION

The typical actual titration proceeds as follows: A series of doubling dilutions of a given antigen is prepared, beginning usually with a dilution of 1 part of total protein (based on total nitrogen) in 500 parts of buffered saline, pH 7, the buffered saline prepared according to Evans (1922). One and seven-tenths milliliters of each antigen dilution is introduced into each of the ten cells ordinarily used, thus providing a standard series of dilutions of antigen, each of which is half the concentration of its predecessor, covering the range from 1:500 to 1:256,000, inclusive. Each of these antigen dilutions is then placed in the cell-carrier of the photronreflectomer, and the amount of light reflection due to the cell itself plus its contained antigen is recorded. After each antigen control value is thus recorded, 0.3 ml. of antiserum is added, the contents are mixed, and the cell is placed in the air incubator to be read 20 minutes later. The net turbidity is equal to the total turbidity minus the sum of the antiserum and antigen control values.

After a series of ten or eleven cells has been read, the cells are washed with distilled water and allowed to drain for a few minutes, after which they may be used again. If the range of dilutions of antigen from 1:500 to 1:256,000 covers the entire reactive capacity of a given antiserum, its complete titration with one antigen requires about 40 minutes. If, however, the prozone has not been reached with zero reading at the concentration of 1:500 of antigen, supplementary, more-concentrated antigen dilutions must be used. Similarly, if the turbidity has not fallen to zero with a 1:256,000 dilution of antigen, higher dilutions of antigen will be necessary to complete the titration of the particular antiserum and antigen tested. On the average a complete titration requires about 1 hour.

PLOTTING THE RESULTS

The completed titration of a given antiserum and various antigens can be expressed (1) as the individual net galvanometer readings, (2) as the arithmetic summation of the net turbidities throughout the reaction range, (3) in the form of curves, and (4) by calculation of the relative areas under the curves. The results of a typical titration are shown in Figure 1 to illustrate directly the third and fourth methods of expression listed above. Actually, Figure 1 gives also the net turbidity readings which can be read from the curves and the arithmetic summation of the relative turbidities, which is proportional to the areas.

THE INTERPRETATION OF THE DATA

The following considerations apply to the interpretation of these data:

1. It has been well established that the intensity of precipitin reactions is affected by at least these factors:
   a) Amount of antibody and manner of preparation of the antiserum

b) Amount of antigen and manner of its preparation
c) Absolute and relative concentration of these primary reagents
d) pH of the reacting mixture
e) Temperature
f) Time of interaction

2. The precipitin reaction shows the phenomenon of optimal proportions, i.e., certain proportions of antigen and antibody give a maximum turbidity, while relatively more or less antigen than the optimal amount yields less and less precipitate.

3. In the comparison of antigens no single point on the titration curve is any more decisive than any other and nothing less than the whole curve can give an adequate basis for the comparison of a variety of antigens.

4. All results are *relative* to the values obtained in the homologous reactions. By their very nature, comparisons of sera and the relationships of animals are *relative*. The homologous reaction is the standard for each antiserum and the basis to which all heterologous reactions with the same antiserum are referred. The constant source of illumination in the photronreflectometer guarantees that the basis of comparison of the galvanometer reading is a true one, provided the antigens are presented in comparable amounts and physical and chemical states.

In our serological comparisons we have been primarily concerned with the determination of the relative similarities of antigens. In making such determinations each antiserum is tested with a variety of antigens, and the assumption is that the antigens are serologically and chemically similar to the homologous antigen in proportion as they react with this particular antiserum. That this assumption is generally valid is shown by the researches of Landsteiner and others presented in detail in Landsteiner (1936) and Marrack (1938).

The factors which affect precipitin reactions in the comparison of related antigens are controlled in our procedure by preparing antigens and antisera in the single manner appropriate for each and by keeping the pH, temperature, and duration of the reaction constant. Under these conditions the animal producing the antiserum is the greatest variable, for in our experience different rabbits injected with comparable amounts of the same antigen may yield antisera of varying strength, specificity, and reaction range.

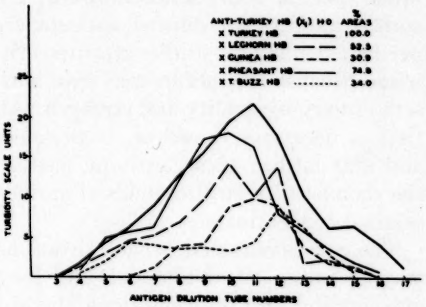

FIG. 1.—The reactions of an undiluted antiturkey hemoglobin serum with standard dilutions of the hemoglobins of turkey, chicken, guinea hen, pheasant, and turkey buzzard. The galvanometer readings are given along the ordinate and the increasing dilutions of antigen along the abscissa. Tube 3 contains 1 part of hemoglobin to 124 parts of saline, and each succeeding tube or cell contains half as much antigen as the preceding one. The curves are predominantly unimodal. The fact that guinea hen and turkey buzzard react about equally well to the antiturkey serum does not prove any close relationship of guinea hen and buzzard to each other. Actually they are on opposite sides of the turkey locus.

This does not mean that similar antisera in these respects cannot be produced, for often they are. It does mean that if one wishes to use *only* antisera of a particular range or strength or discriminating capacity he must usually select such antisera out of those produced by the various rabbits injected. It has been well established now (Wolfe and Baier, 1938; Wolfe, 1939 and earlier) that a short injection series tends to give more discriminating antisera of narrow range, whereas prolonged immunization tends to give less discriminating antisera of broader range. Obvi-

ously, for the determination of distant relationships, such as those between representatives of different phyla, antisera of wide range must be used. These are generally not discriminating enough to distinguish between the homologous species and closely related heterologous species. For the discrimination of closely related species, short-series antisera, absorbed antisera, or diluted antisera are needed. Serological studies are often criticized because the rabbits may give antisera of varying quality and strength. All that is necessary, however, is to select and standardize these antisera, just as the chemist standardizes acids of various original concentrations.

The curves obtained in such titrations may be unimodal or bimodal, and they may occupy any position along the abscissa *depending on the relative strengths* of the antisera and antigens. If the curve is complete, i.e., touches the abscissa at both ends, then the area under the curve is proportional to the summation of points, and various curves obtained with the same antiserum tested with different antigens can be compared by simple summation of points. If the antiserum is relatively powerful and the antigen is relatively weak, then the curve may not descend to the abscissa even with undiluted antigen, and the titration cannot be completed *at that dilution of the antiserum*. Such antisera may be diluted, however, which brings the antigen into relative excess and makes it possible to titrate such an antiserum completely at some higher dilution of the antiserum. Of course, if an antiserum is diluted for this purpose, it must be used in the same dilution for all antigens tested.

The method of plotting employed here is only one of many which could be used. Giving the turbidity in scale units as indicated by the photronreflectometer along the ordinate in arithmetic series and the dilutions of antigen along the abscissa in the geometric series of dilutions employed makes it possible to express the results graphically with a minimum of conversion and in a convenient form. Our basis for the comparison of various curves is not any individual reading for some particular antigen dilution but the whole area under the curve. Values proportional to these relative areas are obtained most readily by simple summation of the readings for all the cells where the curves are complete. The reason for using the sum of turbidities over the whole reaction range is that there is in most cases no single antigen dilution which hits corresponding points on all the curves. Furthermore, it would be difficult to find the exact peak of such curves and the exact end points; but such determinations are unnecessary for these comparisons, since they would change the sum of turbidities but little.

The temperature employed for incubation (37.5° C.) is the common one for serological studies, and the time of incubation for antisera prepared in rabbits was chosen at 20 minutes. Although this does not give the maximal turbidities resulting from the interaction of antigen and antibody, it is sufficient time so that the rate of increase in precipitation has fallen to a low level, and yet rarely is there any evidence of granulation or flocculation of the precipitate.

RESULTS

TYPICAL RESULTS WITH SIMPLE (RELATIVELY HOMOGENEOUS ANTIGENS)

The chief types of curves obtained in such reactions are shown in Figures 1 and 2. As already stated, they may be unimodal or bimodal. In the latter case the evidence indicates that at least two kinds of antibody have been produced in re-

sponse to the injection of the presumably pure antigen. The existence of a unimodal curve does not guarantee that only a single type of antigen and of antibody are concerned in that reaction system, for there may still be two or more types of antigens or of antibodies or of both whose optimum proportions come close to each other and thus, acting cumulatively, yield a unimodal curve.

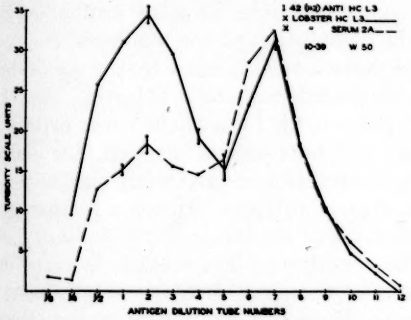

FIG. 2.—The reactions of an antiserum resulting from the injections of purified lobster haemocyanin, with the same antigen in native (serum 2*A*) and purified (HCl_3) condition. Galvanometer readings along the ordinate, standard antigen dilution series along the abscissa. Tube 1 contains 1 part of protein in 499 parts saline, and each succeeding tube or cell contains half as much antigen as the preceding one. The bimodality of both curves is an indication that at least two types of antibody were produced in response to the injection of this purified antigen prepared by Dr. J. B. Allison, Rutgers University. The purification has actually made the antigen more reactive with one of these types of antibody than the native serum was. Six points on these curves were obtained in duplicate. The magnitude of the errors is shown by the length of the vertical bars connecting the duplicate readings.

An illustration of this may be found in Figure 3. Here two distinct reaction systems—anti-*Limulus* tested with *Limulus* serum and anti-*Callinectes* tested with *Callinectes* serum—were tested separately and then in combination. The two modes approached each other in the combination test showing the cumulative effect of the precipitations of the two systems. Of course, this simple summation would not apply to two systems one or both of which was capable of reacting with some component of the other system. The fact that these were actually distinct systems was proved by the testing of anti-*Limulus* with *Callinectes* serum and anti-*Callinectes* with *Limulus* serum, which tests gave no reactivity.

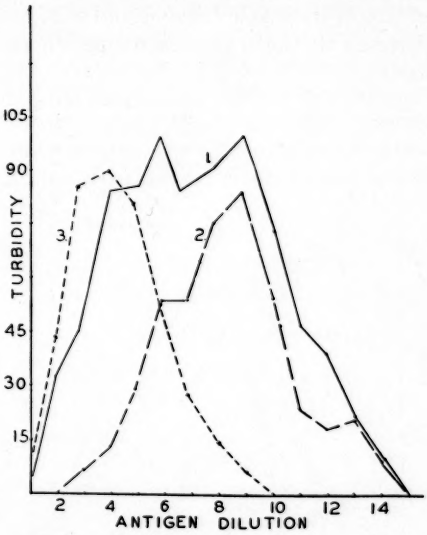

FIG. 3.—The reactions of two distinct precipitation systems first taken separately and then combined. In combination the two systems act cumulatively to give a summation of turbidities. The depth of the valley between the modes has been almost completely filled as the combination titration approaches a unimodal curve. Complete unimodality can thus arise out of a composite precipitation system given suitable values for the antigen concentrations of each.

Curve 1 = the combination of the two systems, area 856. Curve 2 = anti-*Limulus* × *Limulus*, area 441. Curve 3 = anti-*Callinectes* × *Callinectes*, area 416. Each antiserum was diluted with saline when tested separately and diluted to the same degree by the other antiserum when tested in combination.

According to our interpretation, the use of a slightly weaker *Limulus* antigen would have merged the two modes completely, for we have found that, with the antiserum constant, a change in the antigen dilution series merely shifts the posi-

tion of the whole curve along the abscissa. Thus, if the initial antigen concentration is actually 1:250 instead of 1:500, the curve shifts one place to the right; and, on the other hand, if the initial antigen concentration were 1:1,000 instead of 1:500, the whole curve would shift one place to the left, its area remaining the same.

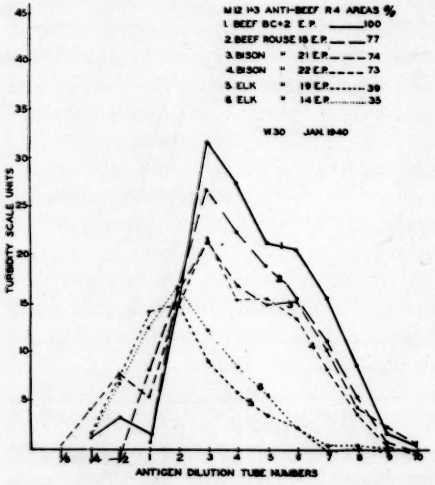
M 12 143 ANTI-BEEF R4 AREAS %
1. BEEF BC+2 E.P. ——— 100
2. BEEF ROUSE 18 E.P. — — 77
3. BISON " 21 E.P. — — — 74
4. BISON " 22 E.P. — — — 73
5. ELK " 19 E.P. 39
6. ELK " 14 E.P. 35
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FIG. 4.—The reactions of an antibeef serum (diluted 1+3) tested with standard dilutions of the sera of (1) domestic cow, (2) Texas longhorn, (3) and (4) two different *Bison*, (5) and (6) two different elk. For the longhorn, *Bison*, and elk sera, preserved with ether-phenol, we are indebted to Charles H. Rouse and the Fish and Wildlife Service. The agreement between the two samples of *Bison* and between the two samples of elk is evident, as well as the predominant unimodality of all the curves. The fact that the longhorn gave 77 per cent intensity of reaction and the *Bison* 74 or 75 does not *prove* that the longhorn was closer to the *Bison* than the other beef sera involved. The values represent the differences and resemblances between the homologous antigen and each of the heterologous antigens but not necessarily the relationship of the heterologous antigens to one another.

TYPICAL RESULTS WITH COMPOSITE ANTIGENS

Mammalian sera commonly induce the formation of antisera yielding unimodal curves (Fig. 4). This cannot be considered as proof of the existence of only a single principal protein in blood sera, for the reasons already given. However, it is probably true that the globulins of mammalian sera are relatively more antigenic than the albumins and that there is, in antisera produced in response to the injection of mammalian sera, a predominance of the antiglobulins. An antisheep serum produced in response to the injection of sheep serum was found to contain twenty-five times as many antibodies to the globulins as to the albumins, the determination being made by the use of the photronreflectometer (Martin, 1942). Whatever the form of the curve expressing the homologous reaction, the form stays the same for all closely similar heterologous antigens. This is a further indication of the biochemical similarity of these antigens. The steady decrease in area of the more distantly related antigens, *Bison* and elk, indicates that there is relatively less antibody in this antibeef serum capable of reacting with each of these antigens. The position of the heterologous curves indicates that each heterologous blood tested had roughly comparable amounts of the corresponding antigens. If this were not true, the various heterologous curves would be displaced farther away from the homologous curve. All the antigens except the first one were obtained in the field by Charles H. Rouse, of the Fish and Wildlife Service, to whom we wish to express our thanks and appreciation. Beef, "Rouse 18 E.P.," was a sample from a Texas longhorn presumably quite remote genealogically from the other beef antigens from near-by domestic cattle. The two different *Bison* samples and the two different elk samples parallel each other quite closely and give as good agreement as can be expected with the sera of different individuals of the same species.

THE EFFECT OF DILUTION OF THE ANTISERUM

Unlike the effect of dilution of the antiserum in the ring test, where considerable dilution may occur before any decrease in titer is apparent, here any dilution of the antiserum is quickly noticed

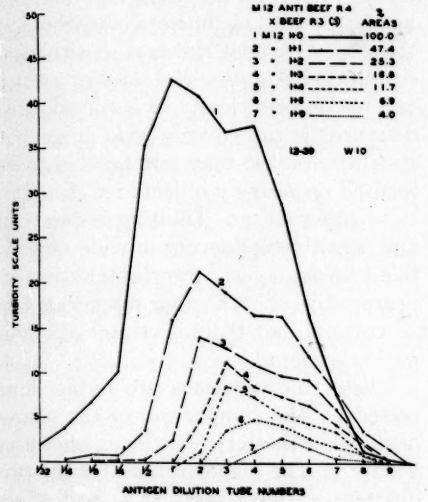

FIG. 5.—The titrations of a powerful antibeef serum *M* 12, against a beef antigen. Each curve represents the complete reactivity of some particular dilution of the antiserum with the standard antigen dilutions. Note the shift of the peak to the right and the great effect of the dilution of the antiserum on the size of the curves.

(Figs. 5, 6). Not only does the area under the curve fall rapidly, but there is a characteristic shifting of the peak toward the right, with a lengthening of the prozone due to the increase in the relative excess of the antigen which must follow dilution of the antiserum. The rate of decrease in area following dilution of the antiserum varies with different antisera and may even be more rapid than shown in Figures 5 and 6, for the reduction is caused not only by a decrease in the amount of antibody present but by the more rapid decrease in frequency of collisions between particles of antigen and antibody which follows upon the dilution.

Figures 5 and 6 show typical results obtainable on dilution of the antisera tested against standard antigen dilution series. The rapid decrease in the area under the curves is at the same time a clear indication of the sensitivity of the photronreflectometer and a demonstration of the marked effect of the concentration of the antibodies on the size of the curves. Furthermore, the rate of decrease in reaction is greater for the heterologous an-

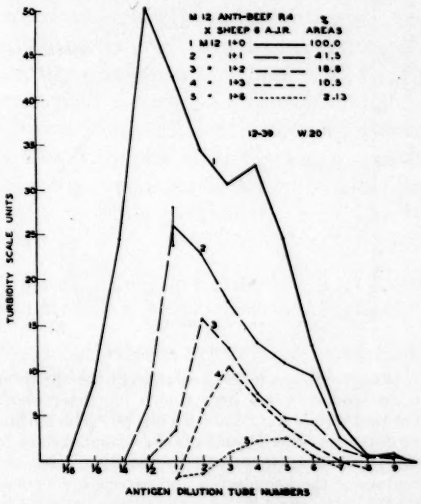

FIG. 6.—The titration of a powerful antibeef serum (*M* 12) with sheep serum. Each curve represents the complete reactivity of some particular dilution of the antiserum with the standard antigen dilution series. Note that the rate of decrease of the curve sizes with the dilution of the antiserum is more rapid when tested with the heterologous sheep antigen (Fig. 6) than with the homologous beef antigen (Fig. 5).

tigen (sheep) than for the homologous antigen. It has, in fact, been known for a long time that dilution of an antiserum made it more specific, i.e., narrowed its range of reactivity and increased its discriminating powers for closely related antigens, but here is the first accurate quan-

titative expression of this effect. Obviously, the percentage relationship of sheep to beef will vary with antisera of different grades of specificity, and, moreover, the percentage relationship values

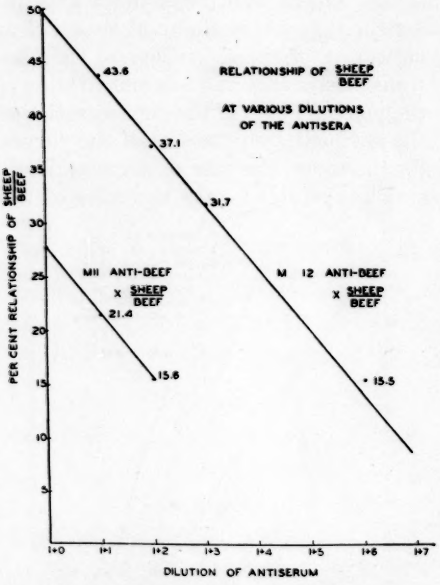

FIG. 7.—The effects of dilution of the antiserum upon specificity are here shown for the powerful antibeef serum *M* 12 and for a less powerful antibeef serum *M* 11. Each point on the diagonal lines for *M* 12 and *M* 11 represents the relative areas in percentage of the homologous and heterologous curves for the particular dilution of the antiserum indicated on the abscissa. Note the regular increase in the capacity of each antiserum to discriminate between the homologous beef and the heterologous sheep sera, that is, the regular increase in the "specificity" of the antisera, with their increasing dilution. The dilution of *M* 12, 1 + 6, gave the *same grade of specificity* as the dilution of the less powerful *M* 11, 1 + 2, that is, both antisera then agreed as to the relative similarity of sheep to beef at 15.5 per cent. Thus, antisera of different original grades of specificity may be made equal by dilution of the less specific ones.

for sheep and beef will vary depending on the *dilution* of *each* antiserum used. We hope that the critics of systematic serology will refrain from saying that because these percentage values depend thus on the conditions they have no reality. It would be just as valid to say, because an object looks big through a powerful telescope and smaller through a less powerful one, that therefore there is no object! The fact is that there is a good analogy between the range and discriminating powers of antisera and those of telescopes. A powerful and sensitive antiserum is like a powerful telescope which can see objects clearly at a considerable distance. It can cover a wide range, but such a telescope may not be able to be focused on near-by objects for clear-cut, close observation. Diluting a powerful and sensitive antiserum of wide range is like focusing a less powerful telescope on nearer objects. A smaller range can now be covered, but there is clearer discrimination of detail.

These considerations are nicely illustrated by the comparison of the effects of diluting these two antisera, shown in Figure 7. The diagonal lines for the two different antibeef sera, *M* 11 and *M* 12, have the same slope, but these antisera differ in their original strength and specificity. The points on each diagonal line represent the percentage areas of the $\frac{\text{heterologous}}{\text{homologous}}$ curves for each of the dilutions of the antisera shown on the abscissa. Thus the percentage relationship of sheep to beef as indicated by *M* 12 varied from nearly 50 per cent when the undiluted antiserum was used to 15.5 per cent when diluted 1 + 6. The antiserum *M* 11, a less powerful antiserum, gave corresponding values of 27 per cent when undiluted and 15.6 per cent when diluted 1 + 2. In other words, a dilution of 1 + 2 for *M* 11 and a dilution of 1 + 6 for *M* 12 gave antisera of the same grade of specificity, i.e., they both gave ± 15.5 per cent relationship of beef to sheep. These results indicate clearly that systematic

serologists need no longer feel helpless before the rabbit, forced to take whatever kind of antiserum it yields. Actually, we can now select and standardize antisera in the sense that we can readily narrow the range to any desired degree. What should be done hereafter, then, is to prepare powerful antisera and dilute them to the desired degree of discriminating capacity. This has the added advantage of increasing the amount of antiserum available from a single injected animal.

COMPARISON OF PHOTRONREFLECTOMETER AND RING TESTS

It is common knowledge that the injection of an antigen into an animal, whether it be guinea pig, rabbit, or horse, does not insure the production of antibodies in that animal. Furthermore, similar injections given to a large group of rabbits will indicate clearly that the response elicited in these animals will, when plotted in terms of titers, units, or precipitating capacities, produce a normal frequency curve. Some animals will not respond at all; others will respond but weakly. The modal group will include the majority of the responders, provided that the class value limits are broad. Finally, there will be present at the end of the curve, if so plotted, the response elicited by the exceptionally good responders.

Unfortunately, this is not the only disconcerting factor involved. Another factor with which many in the field are familiar is that concurrent with the variability in total response is the variability in response to the different portions, fractions, ingredients, and molecular surfaces of the antigen concerned. It is true that the pooling of several antisera made to one antigen would not only obviate the necessity of testing all antigens with all similarly prepared antisera but would also give a truer index of the similarity of the antigens tested than the result obtained in testing with only one antiserum. This is, however, a fairly expensive procedure when an extensive serological program is contemplated. An antiserum made to two series of injections may produce better-"titered" material and may approach the type produced by pooling several single-series antisera, but multiseries antisera are likely to be of little value, if any, in distinguishing between very closely related antigens. Another factor well worth considering is that the component parts of mixed antigens, such as sera, vary with each heterologous antigen. The following values obtained by one of us (DeFalco, 1942), show the variation in albumin/globulin ratios in birds considered to be closely related:

Leghorn.....0.74 (albumin) to 1.0 (globulin)
Guinea hen...3.60 (albumin) to 1.0 (globulin)

Apparently birds are relatively variable in their albumin/globulin ratios, more so than common mammals.

It may safely be stated that almost all phylogenetic serological testing today is based upon doubling dilutions in terms of total nitrogen, total protein, or parts of serum. It is true that comparable dilutions in terms of total nitrogen or protein content is of considerable importance in standardizing the procedure. It is, however, not difficult to test for qualitative differences between any two antigens when the antigenic components are present in equal amounts; but in the testing of many antigens with an antiserum (such as the guinea-hen serum with anti-Leghorn serum) the resulting value is a combination of the quantitative and qualitative differences.

With all these factors in mind concern-

ing differences in response and differences in antigens it is possible to explain why photronreflectometer testing is a better indication of the biochemical similarity of antigens than values obtained by ring test. An actual comparison is shown in Table 1.

TABLE 1

COMPARISON OF PHOTRONREFLECTOMETER AND RING-TEST VALUES

Serum Antigens	Titer 512,000 Anti-Leghorn Serum (to Native) × Native Sera		Titer 1,024,000 Antiduck Serum (to Native) × Native Sera	
	Ring	Photronreflectometer	Ring	Photronreflectometer
Leghorn	100.0	100.0	0.8	.....
Plymouth Rock	100.0	102.1	0.8	28.7
Buff Orpington	100.0	97.9	0.8	.....
Guinea hen	1.6	24.5	0.8	.....
Pheasant	50.0	27.0	0.4	.....
Turkey	50.0	27.6	0.8	20.7
Goose	3.1	10.5	50.0	70.0
Duck	3.1	10.9	100.0	100.0
Turkey buzzard	3.1	11.1	.....	1.6
Pelican	3.1	9.6	1.6	10.3
Gull	0.0	19.5	0.4	.....
Emu	0.4	4.4	0.4	.....

Let us assume for the sake of simplicity that an animal has received one series of four injections of guinea-hen serum given on alternate days and in doubling doses. Furthermore, let us assume that the response has been impartial and that 50 per cent of the antibodies have been made to globulins and 50 per cent to the albumins. When the ring test is used to determine the titer of this antiserum, the reaction should proceed as follows: (1) because the globulins are present in smaller amounts than the albumins, they should react sooner with the antiglobulins than the albumins with the antialbumins, which may have a long prozone because of their greater concentration. In this case it is very likely that the globulins react completely far before the "titer-tube" is reached, in which case the constant amount of antiglobulins present in each tube would be in excess of the small amount of globulin left in the titer-tube. In any case it must be clear, with all the variations mentioned, that the last tube will commonly be the reaction between one antigen and one antibody, namely, in this case, between albumins and antialbumins. If all heterologous antigens vary in their constituent parts—and they do in many cases—then the titer-tube may conceivably represent the reaction with one fraction in one heterologous reaction and with another fraction in another heterologous reaction. The reactions in all tubes but the last are usually ignored in the ring test, because it is thought that they contribute practically nothing to these biochemical studies. Thus, the ring test is a combination of qualitative and quantitative testing. How much is qualitative and how much is quantitative is a task too laborious for routine work to determine.

On the other hand, photronreflectometer studies are quite different. Nitrogen determinations are not necessary if the curves are complete and every dilution contributes to the reaction. An arbitrary dilution of the antigen may be made, say, 1 part of serum antigen to 124 parts of buffered saline; should the total turbidity of the reaction between this dilution of antigen and a constant amount of antiserum be zero, then a dilution of 1:249 and further halving dilutions are made until the reaction has reached a peak and then comes to zero again in the zone of antibody excess. It should be obvious that here the actual amounts of protein present in the reaction are small; it makes *no* difference whatsoever

whether the globulins are 66 per cent of the total nitrogen or only 22 per cent, for the reaction will occur only when the proper dilutions are reached. Ironically enough, this is an excellent quantitative estimation of the qualitative differences between antigens, for a wide range of reactions for each constituent part of the antigen is recorded, the net reaction being proportional to the area under the curve described after plotting the individual turbidities at the various dilutions.

The amount of antigen in each tube of the ring test as commonly performed is one-half that of the preceding tube. Where the relationship value between two closely related forms may be 100 per cent, it is possible that the degrees of reaction in both titer-tubes are quite different and that actually the relationship is only 62 per cent. This condition could not happen with the photronreflectometer, where the formation of precipitate at comparable dilutions is not a point for comparison but the actual estimated turbidities are. It would appear that we may be no more justified in comparing the titer-tubes than the biochemist would be justified in comparing two digestive enzymes by the smallest quantity needed to complete the digestion of a certain amount of substrate. Plotting the curves of reaction with varying amounts of enzyme at constant temperature would surely give him more information for a truer comparison.

Thus, in brief, reading the ring test is reading only one point at the extreme end of a curve of optimal proportions. It is an end point and obviously a weak one. The position of this point depends on the degree of sensitivity of the antiserum and the concentration of the antigen, and comparable points can be determined for a series of antigens only if these are really comparable in concentration. On the other hand, the photronreflectometer reads the whole reaction range, through marked turbidities as well as slight ones; and, as long as we obtain complete curves, the concentrations of the antigens need not be determined, for no more reaction is possible than occurs in a complete curve with a constant amount of antiserum. Obviously, for those antigens capable of giving good turbidities, the photronreflectometer is superior to the ring test.

There appears, however, to be one advantage to the ring test in the determination of the more distant relationships, i.e., the range of an antiserum may be far greater by ring test than by turbidity measurements. Thus the ring test can be of service in attacking the problems of the relationships of animal phyla, as Wilhelmi (1942) has done. As yet the photronreflectometer has seemed unable to react upon such distantly related antigens. To return to the analogy of the telescope, the ring test, properly performed, is adapted to glimpsing objects on the horizon, the photronreflectometer is designed for the critical inspection of near-by objects.

SOURCES OF ERROR IN THE USE OF THE PHOTRONREFLECTOMETER

The photronreflectometer is a delicate instrument in that the galvanometer needle is sensitive to slight disturbances. Thus rubbing the case will often generate sufficient static charge to cause the needle to stick to one position or to give erroneous deviations. Polishing the galvanometer window just before use will not do. Normally, the needle can be set at 50 units, and it will return there after every manipulation, proving the constancy of the illumination.

More important than this as a source

of error is the physical state of the antigen. As yet we cannot guarantee that, even with buffered salines, the corresponding antigens are in a similar condition as to the size and numbers of particles present for a given protein concentration. The correlation of galvanometer readings with sizes and numbers of particles will require careful study. At present we assume that our turbidity readings are statistical summations of the particle populations present and that they are comparable in the corresponding antigens used.

We do know that an old serum antigen may be less reactive and give less turbidity with a given antiserum than a fresh one in comparable amounts, and, furthermore, we have found that a half-hour's heating in the water bath at 56° C. will lessen the reactivity of some antigens. Whether the heating produces rapidly the same effect on the antigen as aging over a period of years remains to be demonstrated. That complement will augment certain precipitating reaction systems and fail to affect others has also been observed. Under these circumstances it would be well to use antigens of comparable age and history or to inactivate them and to run each antiserum with the whole series of antigens tested, at about the same time. A more complete account of the effects of complement in the precipitin-precipitinogen system is reserved for the future.

It is well known that certain lipoids may be widely and apparently erratically distributed among living organisms. If these play any marked role in precipitation systems, the results may be of little significance for systematic serology, for we must have *conservative* inherited traits for our comparisons. The studies so far made with the photronreflectometer show little evidence of such disturbances, though we know quite well (Boyden, 1936) that ring tests have given results with certain Crustacea, clearly indicating disturbances due to lipoidal materials. Our tests to date even with crustacean antigens show no significant differences between antisera made to extracted antigens as compared with antisera made to nonextracted antigens.

SUMMARY

The photronreflectometer has decided advantages over the ring test as a means of obtaining quantitative precipitin data for systematic serology. It quickly and accurately measures the whole reaction range of each antiserum with a variety of antigens. Its data are readily plotted in the form of curves whose relative areas indicate the chemical similarities of the corresponding antigens. A description of the new technique in detail is followed by the presentation and interpretation of typical results with simple and with composite antigens. The data indicate that in order to obtain comparable results with antisera from different rabbits it is not only advisable to inject in the same manner but also to select and standardize the antisera so obtained. In other words, the primary reagents, antigens and antisera used in precipitin testing, must be selected and standardized for the particular task in hand if results of value for systematic zoölogy are to be obtained. The roles of lipoids and of complement in these reactions require further study.

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CAROTENOIDS IN THE DISPLAY AND ECLIPSE PLUMAGES OF BISHOP BIRDS[1]

(Six figures)

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IN SPITE of the greatly increased interest in the carotenoids during the last ten or twelve years, few investigators have given attention to the fact that some of these pigments appear prominently in ornamental plumages. In 1934 Brockmann and Völker published an account of studies of the yellow feather pigment of the canary *Serinus canaria* and of the red, orange, and yellow pigments occurring in the feathers of a number of other birds. In the course of their analysis and feeding experiments they came to the conclusion that the yellow pigment of canary feathers, "canary-xanthophyll," is a decomposition product of lutein, one of the most widespread, in plant and animal tissues, of all carotenoids. They also found that zeaxanthin, if added to a carotenoid-free diet, appears in canary feathers but that carotene, lycopene, and violaxanthin do not. They demonstrated the occurrence of lutein and zeaxanthin in the yolk of the hen's egg. They further reported that canary liver lipids and feathers of the masked weaver, *Ploceus cucullatus*, contain only lutein. In the feathers of wildfire weavers, *Euplectes franciscanus* (*Pyromelana franciscana* syn.), they claim to have recognized a mixture of canary-xanthophyll, lutein, and a red pigment which they consider as another decomposition product of lutein. Captive birds of this species were found to be lacking in the red decomposition product. From the red feathers of several species of woodpeckers they isolated lutein and what appeared to be a carotenoid acid, since it could not be extracted from the

[1] Aided by grants from the National Research Council, Committee for Research in Problems of Sex; grant administered by Professor Emil Witschi.

alkaline alcoholic hydrolysate until the latter had been neutralized with acetic acid.

Adlersparre (1939) succeeded in inducing the development of very pale display feathers in *E. franciscanus* by means of a carotenoid-poor diet. In birds which were fed a diet rich in "leaf xanthophyll, zeaxanthin, and lutein" the plumage color never exceeded the brightest obtainable on the regular diet. He concluded that internal factors limit the intensity of color of the feathers.

Male bishop or weaver birds of this and related species undergo a partial molt, involving only the body feathers, at the onset of the breeding season. In wild *E. franciscanus*, *E. orix*, and *E. nigroventris* males, the regenerated display plumage is red orange or scarlet and black. Wild and captive *E. afra* and *E. taha* males don a golden yellow and black display plumage. All these birds lose the brilliant display feathers during the postnuptial molt and acquire a modest brown and white plumage identical with that of the females. The latter molt only at the close of the breeding season. If males of *E. franciscanus*, *E. orix*, and *E. nigroventris* are kept in the laboratory, the display plumages acquired in captivity show distinctly yellow and orange colors in place of the natural red and scarlet. The yellow species of the same genus (*E. afra* and *E. taha*) undergo no appreciable change. It was mainly the purpose of the present study to find an explanation and, as far as possible, a chemical basis for the striking difference in color between the display plumages of wild and captive males of *E. franciscanus*, *E. orix*, and *E. nigroventris*. The color reaction of these captive birds may be an expression of a relationship of fundamental nature between decreased sexual vigor and some specific nutritional deficiency.

The author wishes to express his appreciation to Dr. Emil Witschi for suggestions and guidance given during the course of this investigation.

FEATHER ANALYSIS

Wild E. franciscanus.[2]—The red feathers of skins of wild *E. franciscanus* males collected during the breeding season in the natural habitat were removed and hydrolyzed with 10 per cent KOH in 85 per cent ethyl alcohol for a few hours at 35° C. Usually all portions of the feathers except the black melanin granules in the basal down and barbs go into solution. The hydrolysate was extracted with benzene, the soaps, base, alcohol, and other products of keratin decomposition being washed out with distilled water. The benzene solution of the unsaponifiable material was dried with anhydrous $CaCl_2$ and concentrated under reduced pressure. Fractionation of the pigment mixture in the solution was obtained by chromatographic adsorption on a double column, of which the upper portion consisted of $CaCO_3$ and the lower of a mixture of two parts (by weight) of MgO and one part of siliceous earth. The magnesium oxide and the siliceous earth were those recommended by Strain (1934, 1942). The chromatograms were developed by the addition of more benzene, and the fractions were eluted from the column with a 99:1 mixture of benzene and absolute ethyl alcohol. Occasionally the alcohol concentration had to be raised to set free an especially tenaciously adsorbed fraction.

[2] Skins of wild *E. franciscanus* and *E. orix* were obtained through the courtesy of Dr. Ernst Mayr, of the department of ornithology, American Museum of Natural History, New York. The author expresses his thanks to Dr. J. P. Chapin, of the American Museum, and Mr. R. E. Moreau, of the East African Agricultural Research Station, Amani, Usambara, Tanganyika Territory, for a skin of wild *E. nigroventris*.

The alcohol was then washed out of the benzene solution. The absorption spectra of the pigments in benzene and chemically pure CS_2 were recorded with the aid of a Bausch and Lomb Universal Spectrophotometer.[3] Technical benzene which had been percolated through a column of $CaCO_3$ was used.

The results of this analysis are shown in Figure 1, *A*. As is indicated in the benzene solutions by Kuhn and Brockmann (1932), they have always been constant. The difference between the author's determinations and those of Kuhn and Brockmann amounts to a shift of about 15 mμ toward the violet end of the spectrum. The CS_2 maxima of the identifiable plant carotenoids have always been in agreement with those in the compilation by Karrer (1936). There was no

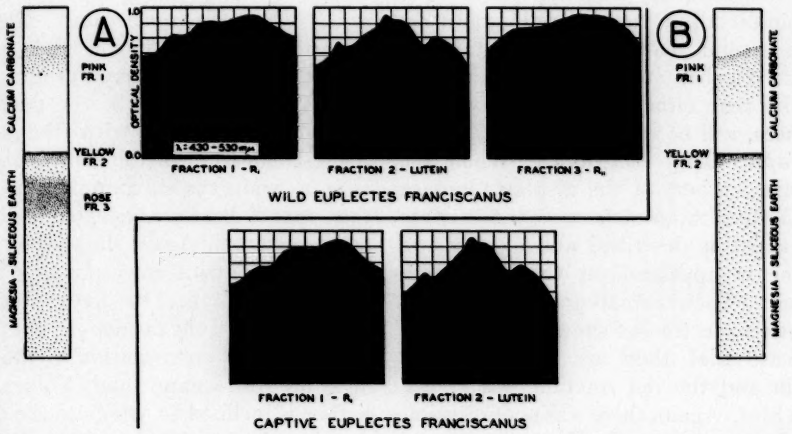

FIG. 1.—Chromatograms and absorption spectra in CS_2 of the carotenoids in (*A*) wild *E. franciscanus* display feathers and (*B*) captive *E. franciscanus* display feathers. The ordinates of the graphs represent optical density from 0.0 to 1.0. The abscissae represent the segment of the visible spectrum from 430 to 530 mμ. When moistened with benzene, the MgO-siliceous earth mixture takes on a brownish color, represented by diffuse stippling, upon which the colors of adsorption bands become superimposed.

chromatogram, three fractions separated on the column. Fraction 3, the least adsorbable of the three, is red; fraction 2 is yellow; and fraction 1 is also red. As shown by its adsorption spectra (benzene maxima at 442, 459, 492 mμ; CS_2 maxima at 445, 473, 508 mμ), fraction 2 is lutein. The author has found lutein in a number of different plant and animal tissues. Although their benzene maxima have never agreed with those given for trace of the canary-xanthophyll described by Brockmann and Völker.

Fraction 3 resembles the red "decomposition" product referred to by Brockmann and Völker, at least in some of its properties. Its benzene absorption maxima consist of a single rise between 465 and 512 mμ with a peak at 480 mμ. In CS_2 it has two maxima consisting of a brief rise at 450 mμ and a broad band with highest absorption at 495 mμ. Fraction 1 of the wild *E. franciscanus* feathers is also red and is characterized by very high adsorbability. Its absorption maxima in benzene are at about 460

[3] The author is indebted to Dr. H. P. Smith, of the department of pathology, College of Medicine, State University of Iowa, who permitted the use of this instrument.

and 485 mμ, while those in CS_2 consist of a slight rise at about 450 mμ and two others at 475 and 505 mμ. Beyond 530 mμ the absorption in CS_2 drops rapidly in the longer wave-lengths, lacking, in this respect, additional peaks which might serve to identify it with any of the better known red polyene-alcohols. For the sake of clarity, fraction 1 will be referred to in the following discussion as R_1, since it is most strongly adsorbed and is found in almost all of the materials examined. The second red pigment (fraction 3), which is characterized by less adsorbability than either of the other two substances, will be referred to as R_2.

Captive E. franciscanus.—When the orange feathers of the display plumage of captive *E. franciscanus* males were extracted as described above, only two pigments appeared on the adsorption column. The chromatogram and the adsorption spectra (as shown in Fig. 1, *B*) indicate that they are identical with lutein and the red fraction, R_1, of the wild bird. Again, there was no indication of the canary-xanthophyll of Brockmann and Völker. The other red fraction, R_2, found in the wild birds' feathers, was absent. This agrees with the corresponding findings of the same authors. Similar analyses (using micromethods of chromatography) of the unsaponifiable liver lipids of captive birds gave identical results.

Since the above observations were not entirely in agreement with the data presented by Brockmann and Völker, it was decided to examine also the carotenoids of canary feathers and of the feathers of several other species of bishop birds and related finches. As the author chanced to obtain a specimen of the red-headed woodpecker, *Melanerpes erythrocephalus*, its red head feathers were also examined.

The canary S. c. canaria.—When canary feathers were analyzed by the methods described above, the chromatograms were practically the same as those of captive *E. franciscanus* feathers. As shown in Figure 2, *A*, two fractions separated on the columns. The absorption spectra of the lower fraction identified it as lutein, while those of the upper indicate that it is similar to, though not identical with, R_1, found in both wild and captive *E. franciscanus*. The benzene maxima consist of a slight rise at 452 mμ and a broad band ranging from 460 to 490 mμ. In CS_2 the maximum (475 mμ) coincides with the second highest elevation of the *franciscanus* R_1 (Fig. 1), while the maximum of the latter (505 mμ) is barely noticeable in the canary spectrum. Again the author failed to find a pigment corresponding to canary-xanthophyll. The liver lipids, as well as those of the fat depots, contained only lutein, a confirmation of the findings of Brockmann and Völker. The author is inclined to attribute the difference in color between the feathers of the canary and those of the display plumage of captive *E. franciscanus* males to the deposition of relatively less pigment in the canary feathers.

The masked weaver, P. cucullatus.—Brockmann and Völker found only lutein in the feathers of this species, whereas the present study indicates the presence, in addition to lutein, of a trace of red pigment with adsorbability and absorption spectra similar to those of the red substance found in the feathers of canaries (Fig. 2, *B*).

The Grenadier weaver, E. orix.—Analysis of the red feathers of a skin of this species collected in the native habitat gives results similar to those obtained for feathers of wild *E. franciscanus*. It was noted, however, before the feathers were

plucked, that the general color of the carotenoid pigmented areas was more yellowish than the same areas on skins of *E. franciscanus*. The chromatogram is similar to that of the wild *franciscanus*. It differs only in that the red band representing R_2 (fraction 3) is lighter and more diffuse. Evidently the mixture contained less of this substance in proportion to lutein and R_1. However, the absorption

The orange feathers of captive males of this species give the same results as those of captive *franciscanus* and *orix* (Fig. 4, *B*). The fact that in the laboratory they are usually quite reddish, and the relatively deep color of the R_1 band absorbed on the $CaCO_3$, suggest that they contain a relatively high concentration of the pink pigment.

The Napoleon weavers, E. afra and E.

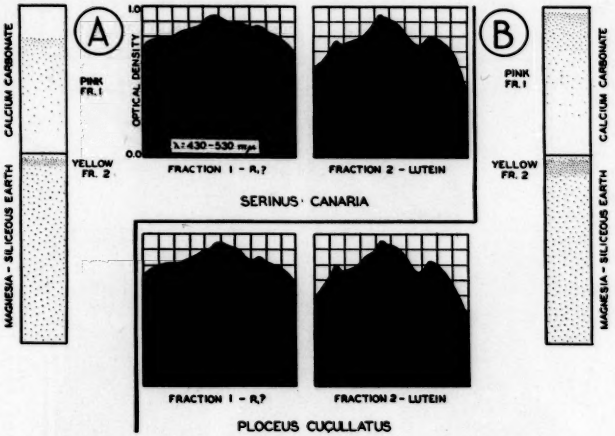

FIG. 2.—Chromatograms and absorption spectra in CS_2 of the pigments found in (*A*) *S. canaria* feathers and (*B*) *P. cucullatus* feathers. Explanations as in Fig. 1.

spectra in benzene and in CS_2 show that each fraction is identical with the corresponding pigment in *franciscanus*. (Fig. 3, *A*).

The orange feathers of a captive bird of this species contain the same mixture of lutein and R_1 as those of the captive *E. franciscanus* males (Fig. 3, *B*).

The black-bellied weaver, E. nigroventris.—The display plumage of wild *E. nigroventris* is not very much more red in hue than that of captive birds of the same species. Yet analysis of one available skin demonstrates (Fig. 4, *A*) that it contains the same three components as the display plumages of wild *E. franciscanus* and *E. orix*. As in *E. orix*, the R_2 fraction is relatively low.

taha.—Analysis of the yellow feathers of captive Napoleon weavers shows that the color is due to the same mixture found in the yellow feathers of the masked weaver, *P. cucullatus*. Most of the pigment is lutein. In addition, there is a small amount of a more adsorbable red pigment, the absorption spectra of which resemble those of the red pigment found in canary feathers. A chromatogram and the CS_2 absorption curves typical of the pigments found in the display plumages of these two species are shown in Figure 5, *A*. The livers and the fat depots contain the same mixture, although in some cases the ratio of the red pigment to lutein is more like that found in the orange-feathered species.

The eclipse plumages.—As has been previously stated, the male bishop birds revert at the time of the postnuptial molt to the henny plumage. Analysis of the tensity of which was never great enough to give an absorption curve by which the identity of the pigments could be ascertained. The amount of pigment yielded

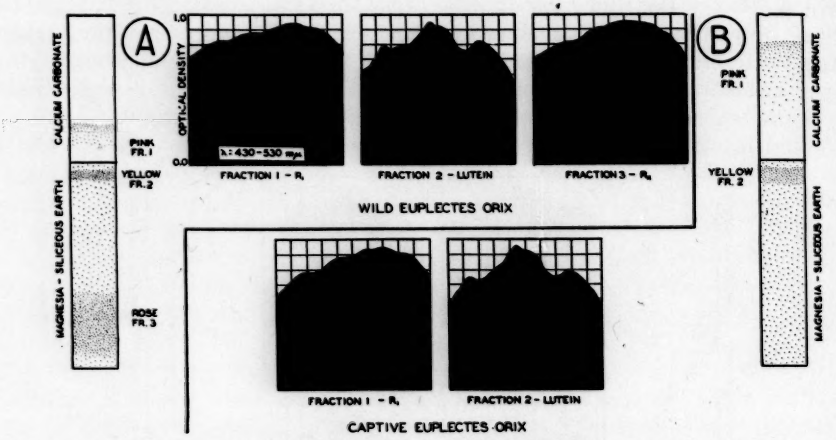

FIG. 3.—Chromatograms and absorption spectra in CS_2 of the pigments found in (*A*) wild *E. orix* display feathers and (*B*) captive *E. orix* display feathers. Explanations as in Fig. 1.

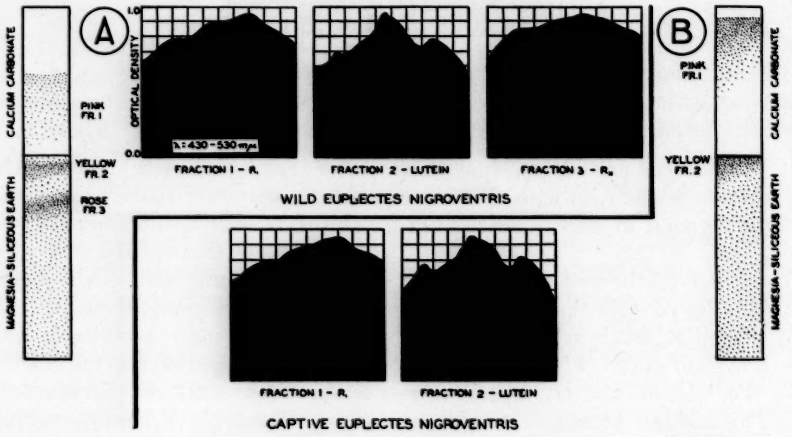

FIG. 4.—Chromatograms and absorption spectra in CS_2 of the pigments found in (*A*) wild *E. nigroventris* display feathers and (*B*) captive *E. nigroventris* display feathers. Explanations as in Fig. 1.

brown and white feathers characteristic of these plumages never went beyond the extraction stage. The unsaponifiable matter of these preparations contained only the faintest traces of color, the in- in such instances was far too small to be studied with chromatography.

The redheaded woodpecker, M. erythrocephalus.—When the red head feathers of *Melanerpes* were examined with the

same methods used in the previously described analyses, the pigment mixture proved to be quite different from that found by Brockmann and Völker in other woodpeckers. Upon extraction of the alkaline alcoholic hydrolysate with benzene, most of the pigment passed immediately into the upper layer. Some of the red pigment, apparently adsorbed on, or in some other manner bound by, the feathers of wild bishop birds (cf. Fig. 5, *B*, with Figs. 1, *A*, 3, *A*, and 4, *A*).

A summary of the data obtained in the analyses of feathers is presented in Table 1.

Seed analysis.—In order to determine the carotenoid content of the regular diet of the captive birds, separate analyses of the component seeds were made. The diet consists of canary grass seed

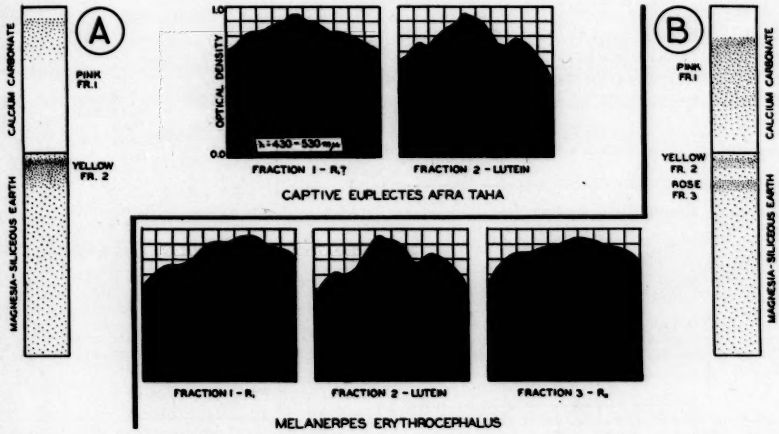

FIG. 5.—Chromatograms and absorption spectra in CS_2 of the pigments found in (*A*) captive *E. taha* display feathers and (*B*) the red feathers of the wild *M. erythrocephalus*. Explanations as in Fig. 1.

soaps formed in the feather decomposition. remained in the lower layer; but, upon repeated extraction with benzene, it was almost entirely removed.

Adsorption on the same type of column as used in the previous analysis gave a chromatogram qualitatively similar to those obtained in analyses of wild *E. franciscanus* and *E. orix* display feathers. It differs quantitatively in that the R_2 and the lutein fractions form only very narrow bands, while the R_1 fraction band is much wider and far more intense in color than the corresponding fraction of the other species. The absorption spectra in CS_2 indicate that these three pigments are identical with those in red

(*Phalaris canariensis*), millet (*Panicum miliaceum* and *Chaetochloa* sp.), black thistle seed (species unknown), and a small quantity of chopped pepper seed (*Capsicum frutescens*) in the proportion, respectively, of 10:5:2:0.2. Two or three pound lots of each type were analyzed separately in essentially the same manner as the feathers.

The seed was finely ground in a hammer mill, and the resulting meal was repeatedly extracted with acetone and ether until no more color could be eluted. The combined extracts were freed of the solvents *in vacuo*, and the oil was saponified under the same conditions maintained in the saponification of the feath-

ers. Usually the process had to be continued for about 24 hours. The alkaline alcoholic solution was then extracted with ether in a separatory funnel. The ether was driven off under reduced pressure, and the precipitate of unsaponifiable matter was taken up in benzene. Fractionation was performed on MgO-siliceous earth columns. The oil of the black thistle seed proved to be devoid of carotenoids. The unsaponifiable matter was colorless. The yellowish-amber color of this oil was assumed, therefore, to be due to the presence of unsaturated fatty acids.

TABLE 1

CAROTENOIDS FOUND IN FEATHERS OF BISHOP BIRDS, THE MASKED WEAVER, THE CANARY AND THE REDHEADED WOODPECKER

Type	Pigments*	Absorption Maxima in Benzene (mμ)	Absorption Maxima in CS_2 (mμ)
1. Wild *E. franciscanus*, *E. orix*, *E. nigroventris*, *M. erythrocephalus*	R_1 Lutein R_2	460, 485 442, 459, 492 465–512 (480 max.)	450, 475, 505 445, 473, 508 450, 495
2. Captive *E. franciscanus*, *E. orix*, *E. nigroventris*	R_1 Lutein	460, 485 442, 459, 492	450, 475, 505 445, 473, 508
3. Captive *S. canaria*, *P. cucullatus*, *E. afra*, *E. taha*	R_1-like Lutein	460, 490 442, 459, 492	475, 505 445, 473, 508

* In each group listed in order of decreasing adsorbability.

The oil of the canary seed contained four pigments: cryptoxanthin, lutein, zeaxanthin, and either flavoxanthin or antheraxanthin. The pigments were identified by their relative adsorption affinity and by their absorption spectra in CS_2. The millet contained α-carotene, lutein, zeaxanthin, and a fourth pigment which was probably flavoxanthin. The chopped pepper seed contained β-carotene, cryptoxanthin, zeaxanthin, capsanthin, and capsorubin. The curves and adsorption properties of the latter two red pigments agree neither with those of R_1 nor with those of R_2. All the pigments found in the seeds were easily eluted from the magnesia by the addition of a little alcohol to the benzene. The absorption maxima in CS_2 of the pigments found in the seeds are given in Table 2.

TABLE 2

CAROTENOIDS FOUND IN THE UNSAPONIFIABLE LIPIDS OF THE SEEDS FED TO THE LABORATORY BIRDS

Seed	Plant	Pigments*	Absorption Maxima in CS_2 (mμ)
White canary seed	*Ph. canariensis*	Flavoxanthin or antheraxanthin	446, 478, 511
		Zeaxanthin	450, 485, 517
		Lutein	445, 475, 509
		β-carotene	452, 482, 517
Red and white millet	*Pa. miliaceum*, *Chaetochloa* sp.	Flavoxanthin	447, 477
		Zeaxanthin	450, 483, 516
		Lutein	446, 475, 511
		α-carotene	475–80, 508
Chopped pepper seed	*C. frutescens*	Capsorubin	467, 503, 540
		Capsanthin	503, 542
		Zeaxanthin	450, 585, 518
		Cryptoxanthin	452, 483, 518
		β-carotene	452, 482, 520
Black thistle seed	(?)	None	.....

* Listed in order of decreasing adsorbability. For carotenoid content of paprika, see Zechmeister (1938).

FEEDING EXPERIMENTS

Effects of a carotenoid-free diet.—Ten birds (three *E. afra*, two *E. franciscanus*, four *E. nigroventris*, and one *P. cucullatus*), all except the specimen of *Ploceus* in practically carotenoid-free eclipse plumage, were placed on a synthetic diet very low in, if not free of, carotenoids during the nonbreeding season.[4] After the birds

[4] Composition of the synthetic diet:

Alcohol-extracted casein	250 parts
Coarsely ground polished rice	450 parts
Cottonseed oil	150 parts
McCollum and Simmonds Salt Mixture, No. 185	50 parts
Ground agar	30 parts
Irradiated 7-dehydrocholesterol	500 U.S.P. units
Vitamin A-ester	4,000 units
Dried brewers' yeast	50 parts

The author is indebted to Dr. J. Waddell, Research Laboratories, E. I. du Pont de Nemours and Company, New Brunswick, New Jersey, for a supply of the dehydrocholesterol.

had been kept on this diet for 2 months, feathers were plucked from areas which are carotenoid pigmented in the breeding season. All except the *Ploceus* then received injections of pregnant mare serum in order to induce regeneration of display feathers in the plucked areas.[5] Witschi (1935, 1936) has demonstrated that injection of various gonadotropic substances causes regeneration of display feathers during the nonbreeding season. In *Ploceus* the plumage contains carotenoid-colored feathers throughout the year; therefore, hormone injections are not necessary in tests with this species.

The *afra* males responded to the gonadin injections by regenerating display feathers of normal yellow color. The *nigroventris* males reacted similarly, though the normally bright orange color appeared much diluted and rather yellowish. *Ploceus* regenerated approximately normal yellow feathers. The *franciscanus* males were disappointing by failure of regeneration at the proper time. When finally new feathers appeared, they were of the eclipse or hen type, which was simply due to the fact that regeneration was no longer kept under the control of gonadin. Evidently the result of the feeding experiment is mainly negative. After nearly 3 months of carotenoid-free diet the yellow fraction appears not perceptibly decreased, and even the red fraction is only diluted but not absent. Autopsies at the end of the experimental period show that rich stores of pigment are still maintained in the liver and in adipose tissues.

It is concluded that, in order to effect a depletion of stores within the birds, carotenoids would have to be withheld from the food for much longer periods, probably over one full year.

Paprika-feeding.—Canary-breeders have long known that a supplement of paprika, *C. annuum*, added to the normal diet of their birds will cause regenerating feathers to acquire an orange color, as opposed to the usual "canary yellow." Since, in the analytical phase of this study, it was found that the pigment complex of canary feathers is similar to that of the captive bishop birds, an attempt to alter the color of the latter birds' feathers by paprika-feeding was made. As the reports of Zechmeister (1937) and Zechmeister and Cholnoky (1938) indicate, the known carotenoids found in paprika are the same as were found by the author in the pepper seed which ordinarily forms a very small part of the regular diet of the bishop birds in this laboratory. The accumulation of the chopped pepper seed on the bottoms of the feeding dishes indicates that the laboratory birds eat little, if any, of this component. Therefore, the addition of paprika to the seed mixture was considered very likely only a means of quantitatively altering the carotenoid complex ingested by the birds.

Eleven birds were used in the experiment. There were four *E. franciscanus* males, three *E. afra* males, and four *E. nigroventris* males. All except one *franciscanus* were in the display plumage, the exception being a bird which was on the verge of undergoing the prenuptial molt. The rump feathers and the tail coverts of all except the molting bird were plucked. Paprika was added to the diet of two of each species. Two each of *franciscanus* and *nigroventris* and one of *afra* were continued on the regular diet as controls.

When feathers had regenerated completely in the plucked areas, they were removed and compared with those which

[5] The author is indebted to the Cutter Laboratories, Inc., for this preparation of the gonadotropic principle of the pituitary (Gonadin).

had been collected at the beginning of the experiment. It was found that those of the paprika-fed *E. franciscanus* were distinctly redder than the control feathers from the same birds. Those birds which were fed the regular diet alone showed no change. No appreciable change was observed in the regenerated feathers of the *nigroventris* or the *afra* males, regardless of what was fed. Continuing the experiment, the paprika was added to the diet of all except one control of each species. After 42 days the feathers were plucked again. It should be pointed out that the supplement of paprika was given in abundance. The birds usually had to pick through a 3-mm. layer of the powdered material to find the seeds of the regular diet. More of the powder was added daily. At the close of the experiment it was found that the regenerated feathers were of usual color in the controls, while in the paprika-fed *franciscanus* and *afra* males they were distinctly reddish. The paprika-fed *nigroventris* males also showed a positive response, though it was not nearly so striking as in *franciscanus* or *afra*.

Chromatographic and spectrometric analysis of the control feathers (feathers plucked from the regular diet-fed birds, as well as those from experimental birds before paprika was fed) gave results identical with the data obtained in the analytical work described above. Only lutein and R_1 were present.

Analysis of the feathers of birds which showed a positive response to paprika-feeding demonstrated that the reddening of the plumage was due to the appearance in the feathers of capsanthin, the red-violet pigment which is primarily responsible for the bright color of paprika and the fruits of other plants of the genus *Capsicum*. This pigment appeared as a red-violet band between the lutein and the R_1 bands. Its absorption maxima in CS_2 lie at 503 and 542 mμ. Only a trace appeared in the feathers of paprika-fed *nigroventris* males, whereas in the *franciscanus* and *afra* males the amount was sufficient to permit easy elution and spectrometry. The red-violet band of capsanthin in these chromatograms cannot be mistaken for the R_2 which occurs in the wild birds' feathers. The latter is less adsorbable than lutein. Since capsanthin is not found in the plumages of wild bishop birds collected in their African habitats, it is evident that R_2 must be still another carotenoid.

Tomato-feeding.—In view of the fact that R_2, which is responsible for the difference in color between captive and wild *E. franciscanus*, *E. orix*, and *E. nigroventris*, bears some resemblance to lycopene in its adsorbability, the feeding of tomato as a supplement was tried on one *P. cucullatus*, two *E. taha*, two *E. nigroventris*, and two *E. franciscanus* males. The *Ploceus*, one *franciscanus*, and the two *nigroventris* males were birds which had undergone the carotenoid-free diet experiment described above. The regular seed diet had been restored to all of them after termination of that experiment. They had been fed seeds for about 6 weeks before the start of tomato-feeding.

The principal pigments of tomatoes are lycopene, α-carotene, and β-carotene. Small amounts of lutein and zeaxanthin are reported to occur in this fruit as well. Initial experiments had shown that our birds do not eat raw tomatoes and take only inconsequential amounts of the dried fruit. It was therefore decided to feed bread crumbs mixed with concentrates of the extracted pigments. Fresh and canned tomatoes were extracted with acetone and benzene until they were almost colorless. The solvents were evaporated under reduced pressure, and the

residue was taken up in a small amount of hydrogenated vegetable oil. This was then thoroughly mixed with dried bread crumbs and fed to the birds after yellow and orange feathers had been plucked. Lettuce seed, *Lactuca sativa*, and black thistle seed were given daily, in addition to a very small amount of the regular seed mixture. Brockmann and Völker report that lettuce seed is practically devoid of carotenoids. The birds ate the bread-crumb mixture very readily, consuming larger amounts of it than of the regular seed diet. The fact that the feces assumed a reddish-orange color indicates that large portions of the pigments pass through the intestinal tract without being absorbed.

The yellow-feathered species (*E. taha* and *Ploceus*) showed no response to the tomato-feeding. But the regenerated feathers of *E. franciscanus* and *E. nigroventris* were of a deeper red orange than had ever previously been obtained. One *franciscanus* responded more positively than the other. When compared with a recently collected skin of a wild specimen, the two *E. nigroventris* males proved to have regenerated feathers typical of the wild state. Neither of the two *franciscanus* males regenerated feathers as red as is typical of wild birds of this species, however. It is felt that, if the supplement were given over a sufficiently prolonged period of time, this result might be attained.

Analysis of the yellow feathers of tomato-fed *Ploceus* and *E. taha* showed a condition typical of the same birds on the regular seed diet. However, when the red-orange feathers of tomato-fed *franciscanus* and *nigroventris* males were examined, the three fractions characteristic of wild birds of these species separated in the column. Spectrometry demonstrated that the red band below the lutein zone is identical with R_2, the reddish pigment which so far had been found only in feathers from wild birds. Evidently the failure of many captive birds to maintain certain reds of their natural plumages is due to definite deficiencies in the standard seed diets offered by the pet shops. It is most likely that lycopene or a closely related pigment is the one which by its absence causes the yellowish discoloration of captive bishop birds. It is not yet known from what animal or vegetal source the birds get this carotenoid under wild-life conditions.

DISCUSSION

Comment on the analytical data.—The data resulting from our analysis of wild and captive *E. franciscanus*, *E. orix*, and *E. nigroventris* agree with those for *franciscanus* as presented by Brockmann and Völker, in so far as three pigments were found in the wild birds' feathers and two in the captive. Lutein was found in both the captive and the wild material in both investigations. But there is considerable disagreement in regard to the remaining pigments. For example, the present author has never obtained, in any feather analysis, a pigment identifiable with canary-xanthophyll. Brockmann and Völker report this as a constituent of the pigment mixtures of both the captive and the wild *franciscanus* and as the only pigment found in feathers of canaries on the regular diet. But the author has found, in each instance, a red pigment of properties very different from those of canary-xanthophyll. The latter is described as a yellow pigment with absorption maxima in benzene at 472, 443, and 418 mμ. The R_1 pigments differ from canary-xanthophyll in their absorption spectra sufficiently to render impossible any thought of identifying either with the yellow material found by Brockmann

and Völker. The author feels that this lack of agreement cannot be attributed to differences in technique or reagents. The methods of extraction in both investigations were similar. The calcium carbonates used were probably not obtained from the same manufacturers. It is well known (Zechmeister and Cholnoky, 1938; Strain, 1942) that the effectiveness of adsorbents of identical chemical composition but of different manufacture may vary considerably. But, as this variation is only in terms of relative adsorption strength, only the rate of flow of a mixture percolating through columns packed with these adsorbents would vary. The relative adsorption affinity of each component of a mixture would be constant. For example, lutein will always appear lower than zeaxanthin on an adsorption column, regardless of the nature of the adsorbent or the solvent. It is therefore felt that the use of different adsorbents cannot account for the lack of agreement between the two sets of results.

Brockmann and Völker report the occurrence of another yellow pigment, picofulvin, in yellow and green feathers of certain woodpeckers. Its absorption maxima in benzene (450, 424 mμ) and its yellow color preclude identifying it with either of the R_1 pigments found by the author.

Although there is some similarity between the absorption spectra in benzene of the red decomposition product found by Brockmann and Völker in the feathers of wild *franciscanus* and R_2, the striking difference between their adsorption affinities for $CaCO_3$, relative to lutein, raises doubt as to their mutual identity. The red decomposition product of Brockmann and Völker was quantitatively separated from lutein and canary xanthophyll by adsorption on $CaCO_3$. R_2, on the other hand, has much less adsorption affinity, relative to lutein, for magnesia, which is many times more effective an adsorbent than any alkaline earth carbonate.

The reasons for the discrepancies between the data of the present study and those of Brockmann and Völker remain unknown. It hardly seems possible that differences as great as the two sets of data show would occur in skins of birds collected in the natural habitat. It is unlikely that the age of skins, preserved under the usual conditions maintained in museums, is a factor. The author obtained results from a *nigroventris* skin collected in Tanganyika only four months before the writing of this report qualitatively identical with those obtained from a *franciscanus* skin collected in Senegal more than fifty years ago.

The nature of the red-bishop-bird carotenoids.—The analyses of the seed diet indicate that neither the R_1 pigment found in *E. franciscanus*, *E. orix*, and *E. nigroventris* display feathers, nor the R_1-like pigment found in the yellow feathers of *E. taha*, *E. afra*, *Ploceus*, and *Serinus* is identifiable with any of the carotenoids ingested by seed-fed captive birds. The very high adsorbability of these pigments suggests that they contain in their molecules more oxygen than does lutein. Zechmeister and Cholnoky (1938) show that, as the oxygen content of carotenoid molecules increases, the relative adsorbability becomes greater. The author is, therefore, led to believe that the R_1 pigments are probably oxidative intermediates in the decomposition of one or more of the dietary carotenoids.

In his review of the literature on carotenoids in animal metabolism Zechmeister (1937) points out that the fate of carotenoids absorbed from the digestive tract may be any of the following: (*a*)

storage in the fat depots and liver, (*b*) use in vitamin A production, (*c*) step-by-step oxidative decomposition in connection with energy metabolism, and (*d*) elimination (lactation, yolk formation, feathers and other integumental derivatives, bile).

Although no mechanisms for the oxidation of carotenoids are known, there is evidence for the existence of such mechanisms in the form of derivatives of known carotenoids, which may be intermediates in the oxidation of excess provitamins A or of carotenoids without vitamin A potency. A red carotenoid of very high adsorbability and with absorption spectra unlike those of plant pigments has been found in the yolk of the hen's egg by the author. Large quantities of R_1 were also found in the integument of terrapins of the genus *Chrysemys*. During hibernation these reptiles depend largely upon the combustion of fat for energy production. It is not improbable that the carotenoids which are always associated with the fats, share this function. In organisms in which the carotenoid intake is high, it would seem that a means of decomposing pigment in excess of what could be eliminated by means of lactation, yolk or bile formation, or deposition in integumental derivatives would be necessary.

It is not unlikely that some such mechanism occurs in birds. That the amount of pigment appearing in the display plumage of bishop birds is only a small fraction of what is absorbed during the $11\frac{1}{2}$ months between prenuptial molts is shown by the fact that plucked birds will several times regenerate orange or yellow feathers with undiminished color intensity throughout the breeding season. The day-to-day ingestion of pigments with the seed diet may be sufficient to color the regenerated feathers to maximum intensity, though large stores in the liver and fat may also be drawn upon. It is possible that, in the captive bishop birds, lutein and R_1 circulate through the body at all times during the year and that the feather follicles undergo some physiological change during the breeding season which enables them to receive the carotenoids from the circulating blood. The variations in proportions between lutein and R_1 or the R_1-like pigment found in orange and yellow feathers may be due to specific differences in the ability of the feather germs to use the pigment or to specific differences in the rate at which the oxidative intermediates are formed. In the canary it seems that the R_1-like pigment may be formed in the feather germ itself, since only lutein was found in the liver and fat depots of this species.

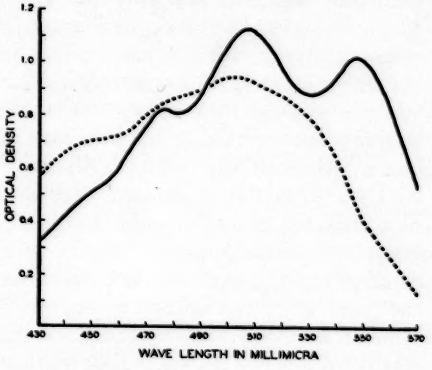

FIG. 6.—Absorption spectra in CS_2 of lycopene (———) and R_2 (·····).

R_2 seems to be a derivative of lycopene. Its absorption spectra are sufficiently dissimilar to those of the tomato pigment to warrant the conclusion that they are not identical (Fig. 6).

It is not difficult to understand why a derivative of lycopene should occur in the feathers of the redheaded woodpecker. Of all the North American wood-

peckers, the diet of *Melanerpes* is perhaps the most varied. According to Forbush (1929), its food "consists of about one-third animal and two-thirds vegetal matter, and both are eaten at all seasons as they can be obtained." Its carotenoid intake may, therefore, be greatly varied, and it is evident that there exists in its diet some source of lycopene or a lycopene-like pigment, probably in berries. Most weavers, buntings, and sparrows subsist largely upon seeds and other vegetal matter in the nonbreeding season but change to a heavy insect diet during the breeding season. This is characteristic of the bishop birds in the wild state, and it is evident that their diet includes plant or animal material which contains lycopene or some very closely related pigment. The fact that the derivative of lycopene found in *franciscanus*, *orix*, and *nigroventris* feathers does not occur in *afra* or *taha* feathers indicates either (1) that these birds are unable to absorb lycopene from the intestine, (2) that the pigment is destroyed before or after absorption takes place, or (3) that their feather germs do not take the pigment from the blood. The inability of the fowl to absorb much, if any, lycopene or other polyene hydrocarbon (Zechmeister, 1937) favors the first alternative. Since much of the red pigment appears in the feces, it appears that none of the bishop birds are able to absorb most or all of the ingested lycopene. It would be interesting to determine whether the higher selectivity characterizing the yellow-feathered species is a primitive or an advanced character.

The appearance of capsanthin in the display feathers of both the red- and the yellow-feathered species after paprika-feeding indicates that they are able to absorb at least two (lutein and capsanthin), and possibly more, polyene alcohols. Adlersparre's failure to obtain an increase in the reddishness of his birds was undoubtedly due to the fact that his experimental diet lacked carotenoids, a condition which did not likewise occur in the regular diet of the controls.

SUMMARY

Analysis of the scarlet feathers of the display plumage of *E. franciscanus* and *E. orix* collected in the natural habitat showed the presence of three carotenoids. These, listed in order of decreasing adsorbability, are a red pigment (R_1), lutein, and a second red substance (R_2). A mixture of the same three carotenoids was found in the red head feathers of *M. erythrocephalus*.

The specific chemical structure of the red pigments is unknown. Instead, absorption spectra of the substances in benzene and in CS_2 are used as means of identification.

Lutein and R_1 were also found in the orange display feathers of captive *E. franciscanus*, *E. orix*, and *E. nigroventris*, but R_2 was consistently missing. This accounts for the more yellowish color of captive males.

The yellow feathers of the canary (*S. canaria* and of three species of yellow weaver and bishop birds contain a mixture of two pigments: lutein and a red pigment resembling R_1 without being identical with it.

Analysis of the diet of the captive birds failed to show the presence of a carotenoid which could be identified with either one of the red pigments. This fact, and its high adsorbability, suggests that R_1 may be an intermediate in the oxidative decomposition of dietary carotenoids. The red substance which accounts for the difference in color between wild and captive *E. franciscanus*, *E. orix*, and *E. nigroventris* is possibly a derivative of lycopene.

Capsanthin appeared in the plumage of paprika-fed bishop birds, but it is not found in the feathers of wild birds.

Tomato-fed *E. franciscanus* and *E. nigroventris* males regenerated display feathers containing the lycopene-like pigment, R_2. It is concluded that the standard diets of captive birds are deficient in lycopene or a closely related substance. The source—vegetal or animal—from which the wild birds get this base material for the R_2 pigment is not yet known.

The failure of tomato-feeding to affect the yellow-feathered species suggests that these are unable to absorb polyene hydrocarbons from the intestine.

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THE RESPIRATORY MECHANISM IN TURTLES[1]

(Nine figures)

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TURTLE breathing presents some unique physiological problems well known to zoölogists. The rigid shell inclosing the viscera prevents general expansion or contraction of the body wall, and a homologue of the mammalian diaphragm is lacking. Certain venerable observers such as Malphigi, Cuvier, and Louis Agassiz (1857) ascribed breathing to throat movements. They reasoned—in part from analogy with the frog—that the hyoid apparatus pumped air into the lungs. Many recent books present this action as the primary breathing mechanism (e.g., Potter, 1938; Walters, 1939; Noble and Noble, 1940).

Mitchell and Morehouse (1863) studied turtle respiration with special attention to anatomical features. Referring to a report on this subject by Townson in 1795, they say in part:

. . . . Having conducted our inquiry with a full knowledge of the opinions of modern au-

[1] I am indebted to Dr. H. F. Prytherch and the United States Fish and Wildlife Service for the use of facilities and the courtesies extended during the conduct of this study at the Beaufort Marine Biological Laboratories; to Dr. F. G. Hall and the Zoölogy Department of Duke University for the use of certain pieces of apparatus; and to the North Carolina State College Research Committee for financial aid.

thorities, we were surprised, on afterward learning the singularly truthful views of Townson, to find they had fallen unappreciated. . . . .

Mitchell and Morehouse have been similarly unappreciated. Emphasis in their studies was given to the anatomy of special musculature for expiration and for inspiration. Throat movements, though functionally unexplained, were removed from consideration in the breathing mechanism. They carefully described the valvate glottis musculature and its elaborate nerve supply. More attention by subsequent workers to this glottis regulation might have saved confusion. Conflicting descriptions of the breathing mechanism are still provoking those interested in turtles (e.g., Pope, 1939; Hansen, 1941).

Various studies of turtle respiration may be classified as follows: (*a*) studies dealing with breathing movements of limb girdles and neck as the primary mechanism (e.g., Wolf, 1933); (*b*) similarly with throat movements (Lüdicke, 1936); (*c*) frequency and rhythm of breathing (e.g., Shaw and Baldwin, 1935); (*d*) studies of gaseous exchange and metabolism (e.g., Hall, 1924; Benedict, 1932). Lüdicke's (1936) paper is of special interest, because he gives evidence for a compromise conclusion. He states that aquatic species (*Emys orbicularis*) swallow air but that terrestrial species (*Testudo graeca*) cannot do so because they are unable to achieve airtight closure of the mouth.

The following studies on turtle respiration involve fuller use of physiological techniques than has usually been the case. The evidence clarifies the mechanism of turtle breathing. Since Lüdicke's work was the most recent to assign a breathing function to throat movements, and since he used a relatively aquatic species, these studies were made on an equally aquatic type, the diamondback terrapin. Certain comparisons were made with other aquatic and terrestrial species.

The diamondback terrapin, *Malaclemys centrata*, inhabits salt and brackish marshes and estuaries. It is distinctly an aquatic type with structural characteristics which make it a good swimmer and diver. It commonly, though not necessarily, swallows its food under water. Except where indicated, all observations in this report were made on this species.

The methods and apparatus for this study varied with the type of observations. They will be described separately under appropriate topics as follows: (*a*) pressure changes in trachea and lung; (*b*) flank-membrane movements; (*c*) throat movements; and (*d*) volume and rate of respiratory exchange. Tracings of records are reproduced in this report.

PRESSURE CHANGES IN TRACHEA AND LUNG

A simple operation made it possible to divide the trachea and interpose a small T-shaped cannula between the cut ends as shown in Figure 1. From the vertical arm of the cannula a piece of gum-rubber tubing was led to a short glass tube. The latter was sealed in a hole drilled through the first costal shield on the left. The specimen from which the accompanying observations were made was thus operated on July 18, 1939, and lived to August 12, 1939. Though there was an unavoidable restriction of the tracheal passage, the animal behaved normally a few days after the operation and continued to do so until a few days before death. Death resulted from a plugging of the cannula, apparently from bacterial growth.

Pressure measurements and records could be made on pulmonary air in this

animal, as shown in Figure 1, and samples could be taken for analysis. Figure 1 shows the outlet connected to a tambour and ink-writing lever on a continuous-feed kymograph and to a manometer, whereby records were obtained of pressure variations during breathing.

A record of pulmonary-pressure changes of a specimen in a tank of water is reproduced in Figure 2. This record shows the following:

a) Pressure in the lungs is independent of atmospheric pressure, and the respiratory system is closed except during expiration and inspiration.

b) Intrapulmonary pressure is nor-

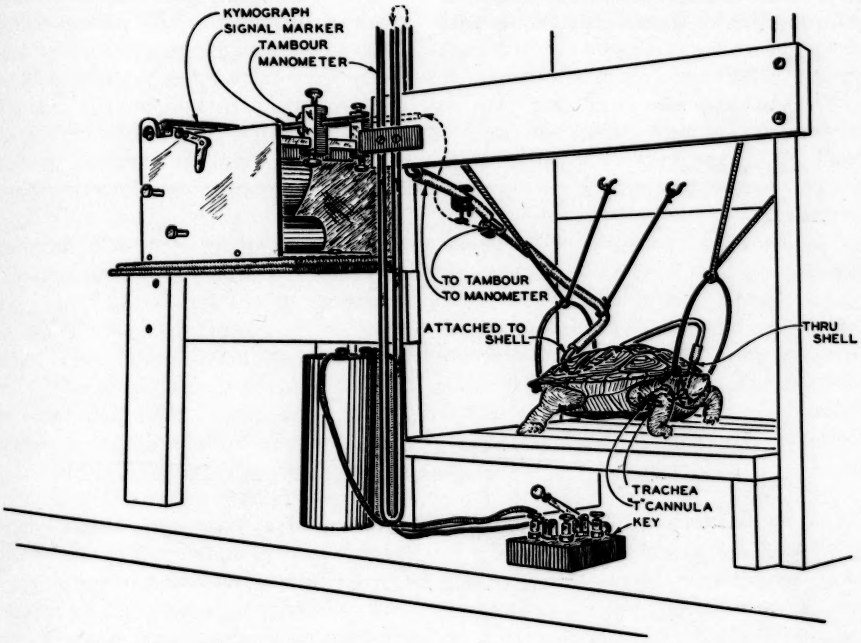

FIG. 1.—Apparatus for measuring and recording intratracheal pressure changes and their correlation with throat and flank-membrane movements.

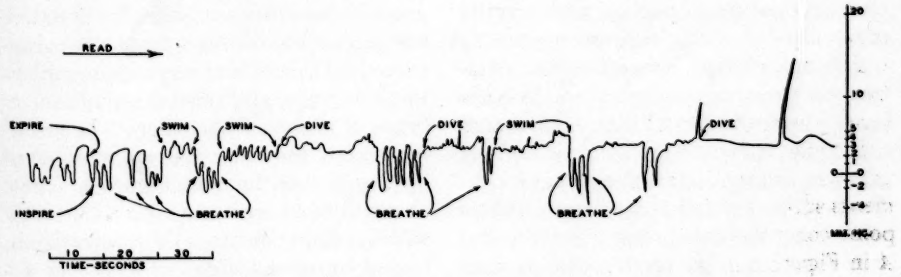

FIG. 2.—Intratracheal pressure changes of a terrapin in a tank of water

mally slightly above atmospheric (2.0–4.0 mm. Hg). Under certain conditions it may momentarily increase considerably above atmospheric pressure (15.0–18.0 mm. Hg).

A manually controlled signal marker was used to indicate the occurrence of throat and flank movements as related to pressure changes. Figure 3 shows the co-ordination of throat movements with

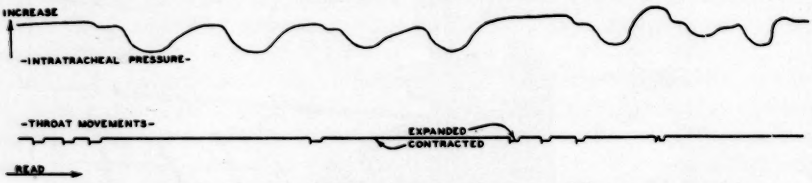

FIG. 3.—Correlation of intratracheal pressure changes and throat movements

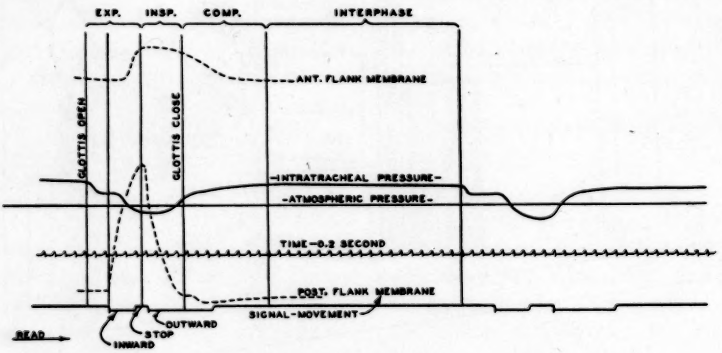

FIG. 4.—Composite record showing correlation of intratracheal pressure and flank-membrane movements. The record is analyzed to show the phases in a resting respiratory cycle.

pressure changes. Preceding this portion of the record was a portion with a series of twenty-five throat movements during which no change whatever in intratracheal pressure was indicated. It is apparent from the record that when actual breathing movements (as indicated by pressure changes) start the throat movements stop. For further evidence on this point compare these results with record *A* in Figure 6 in the section dealing with throat movements.

Similar records were made of the correlation of flank movements and pressure changes. These were later amplified by direct recording of flank movements, and the combined results will be given in the next section (Fig. 4).

FLANK-MEMBRANE MOVEMENTS

The relationship and extent of movement of the anterior and posterior flank membranes was studied with kymographic recordings. Light, counterbalanced levers of the same length were connected by thread and a system of pulleys to an anterior and posterior flank membrane of a normal specimen. The resulting record showed cycles as reproduced by the broken lines in Figure 4. These observations were not made concurrently with pressure changes, but records obtained by methods described in the section on pressure changes made a correla-

tion of these factors possible. That is, while pressure changes were recording, phases in flank movements were indicated on the records by means of the signal marker. Such a record is shown by the solid lines in Figure 4. Therefore, this figure consists of two types of records superimposed for analysis of the events in a single breathing cycle.

The record shows that the anterior membrane does not start inward until the posterior membrane has very nearly reached the maximum inward movement. The anterior membrane moves slightly outward while the posterior is rapidly returning to its outward position, then the anterior returns to its original position. The pressure starts to fall before the posterior membrane starts inward, indicating that the glottis opens before active respiratory movements start. Then, as the membrane starts inward, the pressure rises slightly, indicating that momentarily the air is not escaping as rapidly as displacement of lung space occurs. Then, while this membrane continues inward movement, pressure falls rapidly to atmospheric; then slightly below as the membrane moves outward (correlation not precise on the record), creating a slight negative pressure. Pressure returns to atmospheric as the lungs fill; and just preceding return to atmospheric the glottis must close, for the membrane again moves inward slightly, as though relaxing, and the pressure returns to normal, that is, somewhat above atmospheric.

The correlation of the anterior membrane to the posterior and to pressure changes is such that it can make little active contribution to the breathing cycle. The posterior membrane has an amplitude of movement 4–8 times that of the anterior and has greater area. Thus, the displacement of air in expiration is possible primarily as a result of the movement (passive) inward of the posterior membrane as air is expelled from the lungs; and inspiration must be the result of the active outward movement of this membrane enlarging the body cavity. This movement is analogous to the action of the mammalian diaphragm. However, the anterior membranes and girdle may move in correlation with the posterior membranes to contribute actively to the breathing cycle. This occurs during dyspnea.

The time for one normal cycle was approximately 3.5 seconds at 29° C. These occurred, under the conditions of observation, singly or in series of 2, 3, or more, usually 2 or 3, followed by apnea of 15–60 seconds. When the turtle was active, or following a dive, the dyspneic breathing was characterized by increase in frequency and amplitude of movements, long series of uninterrupted cycles (no apnea), and more active involvement of the pectoral girdle region and sometimes the neck.

THROAT MOVEMENTS

The study of pressure changes in the trachea showed that the throat movements have no function in moving air in and out of the lungs. The evidence was that the glottis is closed except during the inspiratory and expiratory movements of the respiratory diaphragms. That air moves in and out of the nares during throat movements, however, was verified by the use of smoke and wisps of silk. Throat movements also occur under water and move water in and out of the nares. Two essential conditions for the throat action to be a breathing action which aids in lung aeration would be (*a*) a mechanism for closure of the mouth cavity and (*b*) an open glottis at some phase of the movements.

The morphology of the jaws and tongue would appear to make airtight closure of the mouth possible. The tongue is shaped to fit tightly against the posterior nares, and the inner margin of each jaw is a tough pad. These could seal the mouth, but this was not shown experimentally. Aside from its possible importance in breathing, such a mechanism would be of importance in swallowing under water; therefore, its existence need not be associated with throat breathing. Results of a study of the second condition (*b*) were sufficient to decide the question.

It was apparent on direct observation of the glottis that it was closed at all times except during a regular breathing cycle. There was the possibility, however, that forcibly opening or holding open the mouth for observation produced a reflex closure of the glottis. To test this, a small manometer was connected to the throat end of the cut trachea. A by-pass tube was interposed between so that the fluid level in the manometer could be altered at will. Throat movements were found to be ineffectual in producing pressure in the manometer in contrast to Lüdicke's (1936) results from a similar experiment. Furthermore, when a positive pressure was produced in the trachea by means of the by-pass, this pressure was maintained in the manometer until just preceding a regular breathing cycle, when it would release. The glottis must have opened at that time.

Direct observation through the opened mouth of the turtle along with manometer observations showed that the act of forcing the mouth open or holding it open did not interfere with the regulation of the glottis. Therefore, direct observations were made on other species of turtles from various habitats: box turtle, *Terrapene carolina* (terrestrial); loggerhead turtle, *Caretta caretta* (marine); mud turtle, *Kinosternon subrubrum* (aquatic); snapping turtle, *Chelydra serpentina* (aquatic). In every case it was evident that the glottis is closed at all times except during a regular breathing cycle.

It was observed, however, that in a diamondback terrapin under urethane anesthesia the reflex control of the glottis was much less uniform than in a normal specimen. In a normal specimen mechanical stimulation of various kinds, such as tapping the shell, would induce a premature breathing cycle between regular breathing cycles. The specimen became increasingly sensitive to stimuli as the time interval following a cycle increased. It was repeatedly observed that a characteristic response to any stimulus was a breathing cycle. However, in the specimen under urethane, a light tapping of the shell would produce a spasmodic opening and closing of the glottis or a prolonged opening with or without a breathing movement. This suggests that Lüdicke's observation of a positive pressure in the cannulated trachea as a result of throat movements was due to surgical or pharmacological interference with the reflex control of the glottis, which caused it to remain open abnormally. This would invalidate his conclusion that throat movements pump air into the lungs.

The behavior of terrapin in the laboratory suggested olfaction as a likely function of throat movements. It was noticed that before a terrapin bit a stationary piece of food, such as chopped fish or dead fiddler crabs, its nares would be placed almost touching the piece, and a kind of "sniffing" behavior would occur. It made no difference if the food was in or out of water. Moving fiddler crabs, however, were chased and grasped at once. A number of tests were made in which

terrapin, by this sniffing behavior, differentiated between two identical rubber stoppers when crushed fiddler crab was pushed into the hole in one of them. During the interval between breathing cycles an exchange of air in the region of the olfactory epithelium would depend on diffusion if throat movements were lacking. These movements insure an effective olfactory sampling of air and water (taste?). Objective evidence on this point was obtained in the following manner.

A small balloon was held in place under the throat of a terrapin by an aluminum band passed around the head. The balloon was connected to a tambour which recorded its movements on a kymograph, as shown in Figure 5. The stock prevented withdrawal of the head into the shell.

Recordings of throat movements are shown in Figure 6. When the terrapin was free from stimulation, the throat movements during intervals between breathing cycles would become slow, intermittent, and sometimes barely perceptible or lacking. A stimulus such as an electric light turned on, door noisily opened, heavy walking, or sound of conversation would induce strong throat movements, as shown on record *A*, Figure 6.

When undisturbed, the throat movements would increase preceding each breathing cycle, stop during the cycle, and start again after the last inspiration in the series. The pause in throat movements during breathing was invariable (Fig. 6). It was also observed here, as in other experiments, that the general tonus was lowered during a long, undisturbed period of apnea. The head would droop, the eyes close, and the throat movements gradually diminish. In the absence of external stimuli the end of apnea could be predicted when the head would rise, eyes open, and throat movements increase preceding a breathing cycle. This is evidence for a correlation of throat movements with external and internal (CO_2?) sensory stimuli.

Fish and fiddler crabs were used to test responses to olfactory stimuli. Record *B*, Figure 6, shows the results of one experiment. At the point indicated on the record a small piece of wet rubber

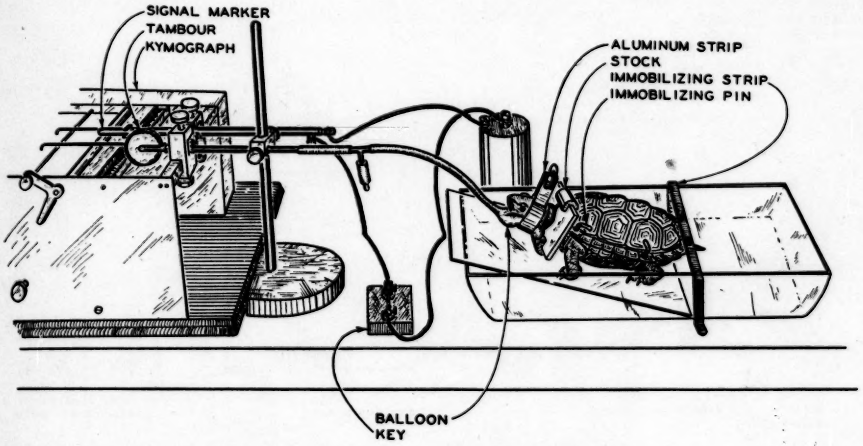

FIG. 5.—Apparatus for recording throat movements

tubing was passed over the nares. The head of the terrapin raised as a result of the general stimulation, but only slight change in the throat movements occurred as compared with the next test. When the rubber was withdrawn the movements diminished. Then the tubing was

Record *D*, Figure 6, shows the results of a test similar to the above except that complicating visual and touch stimuli were eliminated. Two separatory funnels were connected to a single tube which was fixed so that fluid from the funnels would drop just in front of the

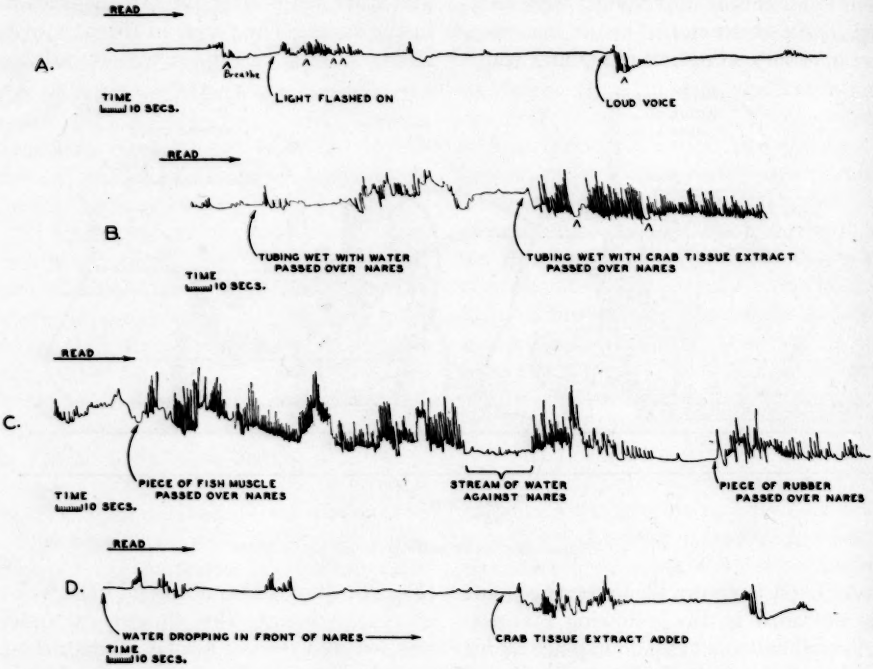

FIG. 6.—Correlation of throat movements with sensory and olfactory stimuli: *A*, response to general sensory stimuli; *B*, response to general stimuli compared with olfactory stimulus; *C*, response to olfactory stimulus compared with general stimulus; *D*, response to mild olfactory stimulus.

wet with crab extract and similarly passed over the nares. A resulting large increase in throat movement is evident on the record.

A similar test with fish muscle and rubber in reverse order from the preceding test is shown on record *C*, Figure 6. When fish was presented, the rapid, deep throat movements which were previously described as sniffing movements are obvious on the record.

terrapin's head. The terrapin and adjacent apparatus were covered with a black cloth. Both funnels were filled with water, and one was used for testing and the other for flushing. Water was allowed to drop from one funnel, and after the initial disturbance the throat movements became subdued, as shown at the beginning of record *D*, Figure 6. Then an extract of crushed fiddler crabs was added to the funnel. The dilution was about

1:400. The results on record *D* show a decided response in movement of the head and increased throat movement. Not apparent on the record are struggling movements of the legs and neck induced by the odor.

These results are believed to show that the throat movements are associated with olfaction as their primary function.

5 mm. I.D. to provide free flow of air. The air inlet to the headcap was bent to dip just below the surface of water in the cap to act as a valve. The headcap was filled with water, except for a small volume of air near the outlet. The cap was elevated in front to cover the diaphragm with water; thus leaks could be detected, and the dead space was at a minimum.

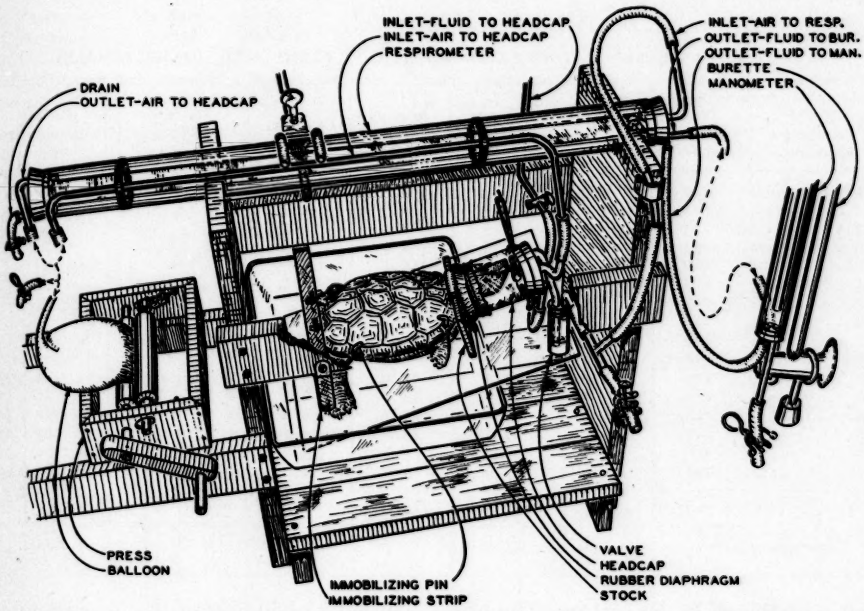

FIG. 7.—Apparatus for measuring various factors in the respiratory gaseous exchange

VOLUME AND RATE OF RESPIRATORY EXCHANGE

The apparatus shown in Figure 7 was used to study the respired air. A stock made from plastic material and brass clamps was used to prevent withdrawal of the terrapin's head, as shown in Figures 5 and 7. The diaphragm of the headcap had to be carefully adjusted to the terrapin's neck so as to prevent leaks but not interfere with breathing. The minimum-size tubing used in the air lines was

With only the headcap in use, expired air was allowed to displace water from a calibrated, inverted tube of 800-ml. capacity. At the same time counts were made of the frequency of breathing. These data are given in Table 1 for a 654-gr. specimen at 29° C. This method, poor for individual expirations, is satisfactory for averaging larger volumes. It was apparent that individual expirations were quite variable in amount, ranging from 6 to 35 ml. More precise data on this

point were obtained with the respirometer to be described.

The respirometer, shown in Figure 7, was designed with the intention of achieving greater accuracy with small volumes of air than that offered by the Krogh or Tissot types. Fluid displaced from the chamber into a 50-ml. burette was equal to the volume of air introduced into the chamber, provided a constant pressure pheric pressure. The balloon was necessary when rebreathing was desired. Since the system had to be of rigid, uniform capacity for purposes of measurement, the press was used to eliminate the balloon while readings were being taken. Checks of the apparatus with known volumes of air indicated a total experimental error of about 1 per cent.

Observations on the volume of air

TABLE 1

FACTORS IN THE RESPIRATORY EXCHANGE AT REST (TEMP. 29° C. 654-GR. FEMALE)

No. of Consecutive Expirations	Total Volume Expired—Ml.	Time—Seconds	Average Volume Ml/Min	Average Volume Ml/Exp	Frequency Exp/Min	Frequency of Cycles in Series				Interval between Series—Seconds		
						1	2	3	4	High	Low	Av.
18	249	290	51.5	13.8	3.7							
14	221	225	59.0	15.8	3.7							
15	223	190	70.5	14.9	4.7							
16	239	290	49.5	14.9	3.3							
16	226	255	53.2	14.1	3.8							
15	219	255	51.6	14.6	3.5							
17	223	275	48.6	13.1	3.8							
19	249	275	54.3	13.1	4.1							
17	229	295	46.7	13.5	3.5							
19	238	320	44.7	12.5	3.5							
19	229	310	44.4	12.1	3.7	0	6	1	1	50	24	33
16	219	278	47.2	13.7	3.5	0	8	0	0	38	24	30
18	228	276	49.6	12.7	3.9	1	4	3	0	59	22	30
18	251	261	57.7	13.9	4.1	0	7	0	1	37	23	29
20	258	330	47.0	12.9	3.6	0	6	1	1	40	24	32
17	228	287	47.6	13.4	3.6	0	4	3	0	52	16	34
274	3,729	4,412	823.1	219.0	60.0	1	35	8	3	276	133	188
Average			51.3	13.7	3.7					46	22	31

was maintained in the system. The burette was suspended by string, pulley, and counterweight so that it was freely adjustable in a vertical direction, and the respirometer chamber was placed horizontally so that displacement of fluid in amounts involved in turtle respiration would not greatly alter the surface level. Thus pressure variations were minimized, and, with the aid of the manometer, the liquid levels in respirometer and burette could easily be equalized for precise measurement of volume. The error due to temperature changes was reduced by resetting each initial reading to atmos-

expired had indicated that considerable variations occurred in successive expirations. This was checked with the respirometer containing slightly acidulated water (with neutral red) to eliminate the absorption of CO_2. The volume of one or two of a series of expirations was determined in each case. Data for a terrapin which had been in the apparatus for 18 hours are given in Table 2.

Some information about the effect of diving on breathing was obtained by subjecting the above terrapin to a simulated dive immediately after the data in Table 2 were obtained. The headcap was

filled with water for 20 minutes. It was then partially emptied, and the first sample expiration was measured within 35 seconds from the end of the dive. These data are given in Table 3.

TABLE 2

VOLUME OF EXPIRED AIR AT REST (TEMP. 26° C. 720-GR. FEMALE)

Number of Expirations	Measured Volume	Average Volume	Interval between Samples
2 in series....	19.3 ml.	9.7 ml.	(Approximately 6 minutes)
1.....	16.1	16.1	
1.....	14.1	14.1	
2 in series....	18.3	9.2	
2 in series....	18.2	9.1	
1.....	16.1	16.1	
1.....	15.3	15.3	
10.....	117.4	89.6	
Average..	.....	12.8	

TABLE 3

VOLUME OF EXPIRED AIR AFTER 20-MINUTE DIVE (TEMP. 26° C. 720-GR. FEMALE)

Number of Expirations	Measured Volume	Average Volume	Interval between Samples
1.....	24.5 ml.	24.5 ml.	35 sec.
1.....	21.6	21.6	3 min.
1.....	18.0	18.0	8 min.
1.....	18.1	18.1	12 min.
1.....	17.1	17.1	20 min.
1.....	16.1	16.1	35 min.
2 in series.....	13.5	6.8	40 min.
2 in series.....	14.1	7.1	50 min.
2 in series.....	17.3	8.7	110 min.
3 in series.....	13.9	4.7	140 min.
2 in series.....	16.6	8.3	200 min.

Resting gaseous metabolism was studied by measurement of the oxygen consumption and by analysis of alveolar air. A 5-N solution of NaOH was used in the respirometer for CO_2 absorption from expired air. The turtle was allowed to rebreathe the air in the respirometer for a measured time, and the reduction in air volume was measured. This would equal the O_2 uptake. The oxygen content of the system was calculated (using data of Wilson, 1939, in part) to be ample for rebreathing periods up to 1 hour.

Results from the same specimen which was used for the preceding observations (Tables 1, 2, and 3) are given in Table 4 for measurement of O_2 uptake. This 720-gr. female terrapin expired 35.0 ml. per kilogram per hour at 24° C. These results are in reasonable agreement with those of Hall (1924) for the turtle *Chrysemys marginata*. Comparing with his results, the CO_2 output for the dia-

TABLE 4

O_2 CONSUMPTION AT REST (TEMP. 24° C. 720-GR. FEMALE)

Burette Readings		Time	Observed O_2	O_2/Hour
Initial	Final			
3.0....	3.0	9:40		
3.0....	18.5	10:15	15.5 ml/35 min	27.6 ml
3.0....	3.0	10:30		
3.0....	20.2	11:05	17.2 ml/35 min	29.6
3.0....	3.0	11:23		
3.0....	20.1	12:00	17.1 ml/37 min	27.8
3.0....	3.0	1:10		
3.0....	14.6	1:35	11.6 ml/25 min	27.1
3.0....	3.0	2:00		
3.0....	16.8	2:32	13.8 ml/32 min	25.8
3.0....	3.0	2:42		
3.0....	16.8	3:20	13.8 ml/38 min	21.8
3.0....	3.0	2:45		
3.0....	15.2	3:15	12.2 ml/30 min	24.4
3.0....	19.7	3:25	16.7 ml/40 min	25.0
3.0....	3.0	7:10		
3.0....	12.1	7:33	9.1 ml/23 min	23.9
3.0....	18.2	7:47	16.2 ml/37 min	26.2
3.0....	21.2	7:55	17.2 ml/45 min	23.0
3.0....	27.5	8:10	24.5 ml/60 min	24.5
3.0....	33.6	8:40	30.6 ml/90 min	20.4
Average	.....	.....	.....	25.2

mond-back terrapin would be estimated to be about 25 ml. per kilo-hour, and the R.Q. about 0.70 at 24° C.

In the study of pressure changes in the turtle lung which was described in the section on intratracheal pressure, a cannula was inserted in the trachea in such a manner as to afford a minimum of interference with normal breathing. With this operation alveolar air was obtained for analysis. A sample was withdrawn from the cannula outlet on the terrapin's back while its head was held under water to prevent entry of air through the mouth or nares. Analyses were made on 5-ml. samples in a Haldane gas-analysis apparatus.

TABLE 5

COMPOSITION OF ALVEOLAR AIR RETAINED IN LUNGS FOR VARYING PERIODS (TEMP. 28° C. 720-GR. FEMALE)

LENGTH OF TIME FROM LAST INSPIRATION	COMPOSITION OF AIR	
	O_2 (Per Cent)	CO_2 (Per Cent)
0.25 min.....	16.46	4.69
1.00 min.....	11.36	6.29
5.00 min.....	10.59	6.83
20.00 min.....	2.56	10.48
40.00 min.....	2.59	12.92

Table 5 gives the results of analysis of an alveolar-air sample taken within 15 seconds following an inspiration and also following arrest of breathing during forced dives of varying lengths. An opportunity to observe large numbers (2,600) of terrapin in the breeding pens at Beaufort, North Carolina, showed this species to spend much time during the day under water. The length of individual dives on July days (room temperature about 29° C.) was generally between 1 and 2 minutes. These terrapin were usually in continuous movement under water. Occasional specimens would lie quietly on the bottom for intervals varying from 3 to 10 minutes.

DISCUSSION

Physiological observations on breathing can be correlated with anatomical structures to explain the mechanism. Figure 8 is a schematic diagram, drawn from dissection, of the special musculature of breathing. Mitchell and Morehouse (1863) describe the precise anatomical details and variations of this musculature for a number of species. These muscles are arranged to act antagonistically in functional groups as illustrated by the models in Figure 9.

Comparison of Figures 4, 8, and 9 will make clear the mechanism of a normal resting cycle. During dyspnea the serratus magnus can contribute actively to the cycle, and this action may be augmented by movements of the pectoral girdle because of the coracoid insertion of these muscles.

The fact that the glottis is closed except during a breathing cycle in the various species examined is important in considering secondary functions of the breathing mechanism. It precludes any respiratory significance for the throat movements in breathing, except possibly a reduction of the dead-air space. It permits the retention of inspired air without tonic contraction of the inspiratory muscles. During sudden withdrawal of the neck and appendages and during dives air may be forced from the lungs. The latter may be passive or active (involving expiratory muscles) and suggests a hydrostatic function. A pressure-receptor function may be postulated for this mechanism in aquatic (particularly marine) species.

The observed power to increase the partial pressure of gases in the lungs somewhat independently of atmospheric

pressure may be important in the respiratory exchange. A compression phase has previously been found in insect respiration (McCutcheon, 1940). Such a mechanism suggests a kind of supercharged respiration. It might have a bearing on between air-breathing animals in which the respiratory epithelium is in continuous pressure equilibrium with the atmosphere (e.g., scorpion, man) and those in which the epithelium is subjected to large physiological variations in pressure

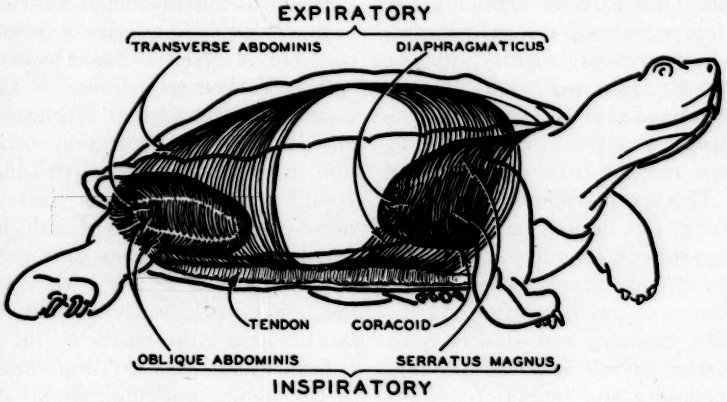

FIG. 8.—Schematic diagram of the musculature involved in breathing

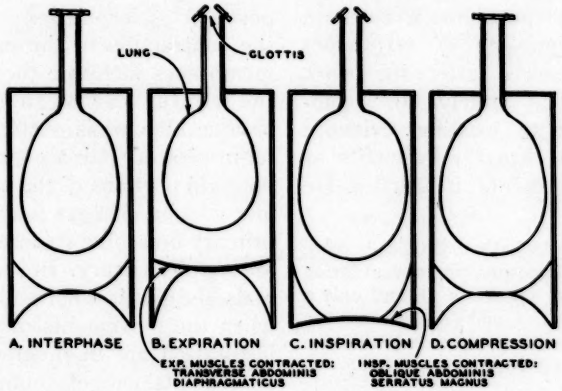

FIG. 9.—Schema to illustrate the essential changes during a breathing cycle

evidence for pulmonary secretion such as reported by Spallita for turtles and Haldane for humans (see Rogers, 1938). In the latter case the bronchioles may duplicate the valvular action of the turtle's glottis.

A distinction can be made at present (e.g., grasshopper, turtle). The former might be termed "homeobaric" and the latter "poikilobaric."

The analyses of alveolar air are in reasonable agreement with those of Wilson (1939) for *Pseudemys troostii* and Vos (after Krogh, 1941) for *Emys orbi-*

cularis with reference to oxygen content after diving (2–4 per cent after 30 minutes). The carbon dioxide findings on the diamondback terrapin are in marked contrast to Wilson's findings on the freshwater species. He simulated breath-holding in this turtle by clamping the trachea for periods up to 33 minutes. The CO_2 increased only slightly, ranging from 3.24 to 4.62 per cent. In the diamondback (Table 5) the CO_2 increased progressively during dives up to 40 minutes, ranging from 4.69 to 12.92 per cent. This species difference, if valid, is interesting, but limited data and different experimental procedures make comparisons unprofitable. The important adjustments and adaptations involving CO_2 capacity and sensitivity in certain other diving animals (Irving, 1939; Scholander and Irving, 1941) indicate that this phase of turtle respiration will repay further study.

Krogh's (1941) published lectures on *Comparative physiology of respiratory mechanisms* appeared after the work reported here had largely been completed. He accepts existing evidence which rules out throat movements as respiratory movements in turtles. He says:

> There is reason to believe [Lüdicke, 1936; Vos, 1936] that the buccopharyngeal ventilation causes no significant uptake of O_2 and only a slight elimination of CO_2, and Vos adduces some evidence (scarcely conclusive) to show that the main function is olfactory.

Vos's paper has not been available to me, and Krogh does not report the evidence to which he refers. My records of throat movements (Fig. 6) offer strong evidence that olfaction is their main function.

SUMMARY AND CONCLUSIONS

A physiological study was made of the mechanism of breathing in the diamondback terrapin. The results were checked with other species, and the general features of the mechanism are considered to be applicable to all chelonians.

The simple breathing cycle consists of an expiration, an inspiration, a compression, and an interval of apnea. These normally occur in a series of two or three consecutive cycles followed by prolonged apnea. Various adjustments of the cycle occur with changing respiratory demands. Repeated expiration and inspiration in series may occur with increased volume exchange and no interval between the series; or there may be infrequent breathing cycles and prolonged intervals of apnea. These will depend on the temperature, activity, and environmental stimulation of the turtle.

Expiration is the result of contraction of the paired muscular membranes inclosing the viscera, the diaphragmaticus anteriorly, and the transverse abdominis posteriorly; inspiration is the result of the contraction of the paired muscular membranes inclosing the flank cavities, the serratus magnus anteriorly, and the oblique abdominis posteriorly. The latter inspiratory pair also provide the main nonrigid portions of the body wall to allow volume changes and constitute the primary breathing structures; the former are supplementary. In contrast to mammals, the glottis is normally closed except when the movements of expiration and inspiration are in progress. The throat movements cannot pump air into the lungs and are not significantly involved in breathing.

Evidence is given which shows that the function of the throat movements is correlated with olfaction.

The respiratory volume was found to vary considerably in a resting specimen. Individual expiration volume was from 6.65 ml. to 34.9 ml., with averages of 12.8–13.7 for 650–720-gr. terrapin at 24°–

29° C. The volume was doubled after a 20-minute dive.

The resting oxygen consumption was found to be 35.0 ml. per kilo-hour at 24° C. Evidence was obtained for a high CO_2 tolerance possibly correlated with diving. After a 40-minute dive the composition of alveolar air was found to be 2.59 per cent O_2 and 12.92 per cent CO_2. Little change in the O_2 content occurred in the last 20 minutes of arrested breathing.

The respiratory system was found to be a relatively closed system in which pressure varied independently of atmospheric pressure. The possibility exists in such a system for active alteration of the diffusion gradient across the lung epithelium. The term "poikilobaric" is proposed for air-breathing animals of this type; the term "homeobaric" is proposed for contrasting types with the respiratory organ in continuous pressure equilibrium with the atmosphere.

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EXPERIMENTAL STUDIES ON THE DEVELOPMENT OF THE CORNEAL REFLEX IN AMPHIBIA. II. LOCALIZED MATURATION OF THE REFLEX MECHANISM EFFECTED BY THYROXIN-AGAR IMPLANTS INTO THE HINDBRAIN

(Three figures)

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IN A previous study on the development of the corneal reflex in Amphibia (Kollros, 1942*a*), it was reported that in all anuran tadpoles the onset of the reflex coincided approximately with metamorphic climax. The reflex always appeared within a period of from a few days before to a few days after the emergence of the forelimbs from the branchial chamber. While in urodeles

[1] This research was done under the direction of Dr. Paul Weiss in partial fulfilment of the require-

the reflex appears even in hypophysectomized animals, which never metamorphose, it is never elicitable in hypophysectomized anurans unless they are artificially stimulated to metamorphose through thyroid administration. In these artificially metamorphosed animals, as in normal ones, the first appearance of the reflex precedes the emergence of the forelimbs by only a few days. This very close association of the initial appearance of the reflex with metamorphosis in anurans suggests that the reflex center is dependent for its maturation upon the metamorphosing agent, thyroid hormone.

Modications of behavior induced by hormone treatment have received considerable attention, much of which has been directed toward studies of the relationship between sex hormones and sexual behavior.[2] Stone (1927), for example, demonstrated that the castration of rats led to a dwindling of their sexual aggressiveness until finally they no longer copulated. Similar studies on rabbits yielded similar results (Stone, 1932). In bilaterally ovariotomized fowl, in which grafts of juvenile testes had been established, Domm (1929) demonstrated crowing, fighting with males, and attempts to tread. Ball (1937) noted that female rats injected with testosterone propionate exhibited masculine behavior. Allee, Collias, and Lutherman (1939) injected normal hens with testosterone propionate. They noted an increase in aggressiveness, initiation of crowing, and, in three hens, initiation of courtship behavior. Shortly after the treatments stopped, these changes disappeared.

Hormones other than those of the sex glands have also been shown to influence the central nervous system and behavior; for example, prolactin induces broodiness in the jewel fish (Noble, Kumpf, and Billings, 1936) and in the domestic fowl (Riddle, Bates, and Lahr, 1935). Thyroid hormone has a marked influence upon the human nervous system. In hyperthyroid states the individual is irritable; in hypothyroid conditions, sluggish; the two conditions also have opposite effects upon the sympathetic nervous system, as revealed by vasomotor and peristaltic activity (Lerman, 1941). Recently Ross and Schwab (1939) discovered that the rate of the alpha rhythm of brain waves obtained from the posterior part of the skull, as recorded by the electroencephalogram, is low in myxedema and returns to normal upon thyroid administration. The correlation between the alpha rate and the state of metabolism was found to be good.

Hormones are capable of modifying not only certain established behavioral traits or of initiating new ones in adults but also of hastening the onset of as yet undeveloped behavioral traits in the young. Domm (1937) injected male chicks with hebin, beginning at the age of 2–4 days. After six injections the chicks crowed, and in some cases they were treading by the thirteenth day. Shortly after the injections were stopped, these adult masculine traits disappeared. A similar precocious establishment of adult traits is brought about by thyroid administration to tadpoles. This results in a premature metamorphosis (Gudernatsch, 1912), including the associated behavioral changes incident to life on land. Among these is the lid-closure, or corneal, reflex.

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[2] See Stone (1939) for an extensive review.

It was the purpose of the present experiments to determine whether or not thyroid hormone is directly responsible for the maturation of the center for the lid-closure reflex. Since thyroid treatment of the animal as a whole results in general metamorphosis, it cannot be determined whether the maturation of the center depends directly upon the hormone or whether it depends secondarily upon hormone-induced changes in some other structures, much as the development of the tympanic membrane, for example, depends upon the formation of the annular tympanic cartilage (Helff, 1928). To prove a direct effect of thyroid hormone upon the reflex mechanism it would be necessary to treat the reflex center alone; if the reflex were to appear prematurely, unaccompanied by any other metamorphic changes, the efficacy of the hormone in bringing about the maturation of the center would be demonstrated. It might be possible to obtain such local effects by implanting pellets of agar, previously soaked in a thyroxin solution, into the fourth ventricle of the medulla oblongata in the region of the reflex center. The local high concentration of thyroxin could be expected to speed the maturation of the reflex mechanism. Implants of thyroxin-soaked agar were first used by Hartwig (1940) to induce local metamorphosis of the dorsal skin and the tail fin of *Salamandra* larvae.

MATERIALS AND METHODS

Tadpoles of *Rana pipiens*, nearly all of which were in mid-larval stages or older, were used. The tadpoles were kept in individual finger bowls and were fed on boiled lettuce. Tactile stimuli were applied with a series of fibers. In all cases the fiber was applied with its side rather than its tip. The stiffness of the fibers was graded according to the method described by Weiss (1942). The tip of the fiber was held against the edge of a pan of an analytical balance, and the counterweight necessary to bend it by about 15° was determined. The values for fibers 1–4 were 3, 18, 57, and 300 mg., respectively. Four modes of stimulation were used: (1) single-point contact, (2) repeated touch to the same spot (temporal summation), (3) single stroke over a larger area (spatial summation), and (4) repeated strokes (temporal and spatial summation). Stimuli were applied to the cornea alone in these tests.

All stimulations were performed under the low power (20×) of a wide-field binocular dissecting microscope. The tadpole remained under water and was unrestrained during stimulation. By the term "metamorphic climax" is meant the period of greatest metamorphic activity, the start of which in anurans can be arbitrarily set as the time of forelimb emergence from the branchial chamber (hereinafter referred to as "f.l.e."). The period immediately preceding metamorphic climax will be termed the "premetamorphic" period or stage.

The tadpoles were anesthetized in a 2 per cent solution of ethyl urethane and were operated upon in amphibian Ringer's solution. A lengthwise cut of 3 or 4 mm. was made through the skin in the midline just dorsal to the medulla oblongata. The edges of the cut were pulled back by small retractors. In the older tadpoles a thin plate of cartilage lies just above the medulla, while in the younger ones the cartilage has not yet formed and only a thin layer of connective tissue lies above the medulla, stretching across between the two lateral masses of muscle. After the cartilage or connective tissue was removed and the muscles on either side pulled back, the chorioid plexus could be seen roofing over the fourth ven-

tricle. A slit about $\frac{3}{4}$ mm. long was made in the chorioid plexus; the blood from this wound was blown away by a jet of Ringer's solution from a small pipette. The bleeding usually stopped in from 10 to 20 seconds. A thyroxin-soaked agar pellet was then introduced into the fourth ventricle through the slit previously made. The overlying tissue was permitted to fall into place, and the edges of the skin were apposed. A small clot usually formed under the edges of the cut. Shortly after recovering from the anesthetic, the tadpole was transferred into fresh well water.

Thyroxin crystals (E. R. Squibb & Sons) were dissolved in slightly alkaline water to furnish the stock solutions in concentrations of 1:500 and 1:1,000. The agar pellets were cut from a larger block of agar. The length of the pellets was never greater than 0.5 mm., while in width and thickness they ranged from 0.1 to 0.5 mm. These pellets were placed in the thyroxin solution for about $\frac{1}{2}$ hour. Shortly before being implanted they were placed on a dry glass plate and permitted to harden slightly. The harder surface prevented their being crushed in subsequent manipulations. In some cases fragments (usually several follicles) of fresh rat thyroid gland were substituted for the thyroxin-soaked agar pellets.

EXPERIMENTAL

In Part I of this study (Kollros, 1942*a*) twenty *R. pipiens* tadpoles had been treated by adding thyroxin to the water (diffuse treatment), with the effect that metamorphosis was markedly advanced. The results of this experiment are shown graphically in Bars II and III of Figure 1. Normally the initial appearance of the reflex precedes f.l.e. by an average of 4 days, while in the thyroxin-accelerated animals, whose metamosphosis had been advanced by as much as 2 or 3 weeks, the onset of the reflex preceded f.l.e. by only 1 or 2 days. If metamorphosis was advanced by about $3\frac{1}{2}$ weeks or more, the onset of the reflex followed f.l.e. by from 1 to 4 days, and in some animals which died on the fifth day after f.l.e. it failed to appear at all. If thyroid hormone does influence the maturation of the reflex center directly, its presence in supernormal concentration, diffusely applied, certainly advances the maturation of the center somewhat less than it does metamorphic changes in body structures, as, for example, the opercular perforation, skin-pigment changes, tail resorption, and so on.

As was indicated before, the implantation of thyroxin-soaked agar pellets into the fourth ventricle might be expected to produce a premature appearance of the lid-closure reflex, owing to the local accelerating action of the hormone on the maturation of the reflex mechanism. In addition, as the thyroxin enters the blood stream by diffusion, a general effect on the animal as a whole might be expected. Because of the lower concentration of the thyroxin in the blood as compared to that in the immediate vicinity of the graft, including the reflex center, the general metamorphic progress of the animal could be expected to be slower than that of the center.

As the onset of the reflex normally precedes f.l.e. by an average of 4 days (Fig. 1, Bar I), any appearance of the reflex preceding f.l.e. by an average of more than 4 days would indicate a stronger local effect. Actually, since diffuse thyroxin treatment displaces the onset of the reflex to a later stage in the metamorphic sequence (Fig. 1, Bars II and III) and as the general influence of the implant on the body generally would tend to do likewise, the relative acceleration of the on-

set of the reflex as compared to f.l.e. will be even greater than recorded.

Series A.—In this series either agar pellets previously soaked in a 1:1,000 solution of thyroxin or fresh rat thyroid fragments were implanted in the fourth ventricle of *R. pipiens* tadpoles at various mid-larval stages. The capacity of rat thyroid implants to stimulate metamorphosis in *Rana* had already been demonstrated (Little, 1929), and the fate of such grafts in *Triturus* had been studied by Idzkowsky (1932). The potency of implanted mouse thyroid in stimulating molting in thyroidectomized *Triturus* had also been tested and the replacement of the implants by host tissue observed (Adams and Merwin, 1939). In the present series only fiber 2 was used as a test stimulus. The first appearance of the reflex was determined by stroking the cornea repeatedly, this being the most effective stimulus. Only after the reflex was established were the other modes of stimulation employed.

Twenty-nine control animals were used. In these the reflex preceded f.l.e. by from 0 to 10 days, or by an average of 4.1 days. This compares very well with earlier observations, in which the period before f.l.e. was given as 4 days (Kollros, 1942*a*). This group and the control group of Series C are indicated by Bar I in Figure 1.

Thirty-three animals with implants in the fourth ventricle first displayed the reflex from 1 to 18 days (average 10.5 days) prior to f.l.e. This treated group can be subdivided according to the stage at the time of implantation. Twelve animals which had received the implant at a stage when no more than three toes were indicated on the hind limb developed the reflex at the normal time or only slightly earlier (Fig. 1, Bar IV). Most of these animals showed an initial phase of metamorphic stimulation; but before the reflex appeared they entered a period of metamorphic stasis of about 2 weeks' duration, due apparently to exhaustion of the thyroid hormone content of the implant. These animals had the appearance typical of thyroid-treated tadpoles.

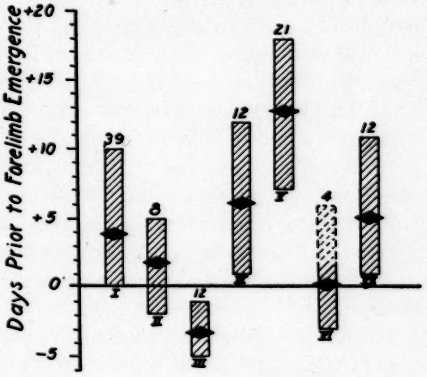

FIG. 1.—The bars represent the time range in days by which the onset of the corneal reflex preceded (or followed) the emergence of the forelimbs. The diamond indicates the average for each group. The number above the bar indicates the number of animals in each group. I, normal controls; II, thyroxin added to water (concentrations less than 1:1,000,000); III, thyroxin added to water (concentrations above 1:1,000,000). IV, agar implants (previously soaked in 1:1,000 thyroxin) in the fourth ventricle of young mid-larval tadpoles; V, similar implants in older mid-larval tadpoles; VI, agar implants (previously soaked in 1:500 thyroxin) in the abdomen of older mid-larval tadpoles; VII, similar implants in the fourth ventricle of older mid-larval tadpoles. The animals represented by Bars VI and VII are from different stocks from those of the other experimental groups.

They remained considerably shorter than their controls, and, in contrast to the latter, their bodies were constricted just posterior to the gills. Development of the hind limbs to an early functional phase had been accelerated and then suddenly terminated. In a few cases incipient changes in pigmentation were noted. Subsequent development to metamorphosis was approximately normal. Little

(1929) observed the same phenomenon of stimulation followed by metamorphic stasis in *R. catesbiana* tadpoles which had received large abdominal implants of rat thyroid glands.

The remaining twenty-one animals of this series were somewhat older at the time of implantation (all five digits indicated on the hind-limb bud). As these animals were more mature at the time of implantation than were those of the previous group, the hormone content of the implant seemed to have been sufficient to permit them to pass through metamorphosis without an intervening stasis period. In many animals, however, the last portion of the period prior to f.l.e. was passed through at a normal rate rather than an accelerated one. In this second group of animals the reflex never appeared less than 7 days prior to f.l.e., with an average period of 12.9 days (Fig. 1, Bar V). The maximum time interval between the onset of the reflex and f.l.e. was 18 days, as compared to 10 days for the controls. The length of the period between implantation and the onset of the reflex was 12 days, on the average, whereas about 20 days would have had to elapse had the tadpoles been subjected to thyroxin treatment at a concentration which would have advanced their metamorphosis to an equal degree.

Ten of the twenty-one animals developed the reflex asynchronously on the two sides. Whereas in the control group the two eyes were never more than 1 day apart in first displaying the reflex, some of the experimental animals showed a difference between the two eyes of as much as 7 days. The asymmetry can be ascribed to an asymmetrical position of the implant, the side of the brain wall nearer the graft being subjected to a somewhat higher hormone concentration than the opposite side. This interpretation is borne out by two cases studied in microscopic sections, in both of which the unilateral precocity of the reflex was found to correspond to proximity between the implant and one brain wall (Fig. 2).

As a further check on the local influence of the implants, four tadpoles received implants of thyroxin-soaked pellets in one of the lateral forebrain ventricles. The metamorphosis of all the animals was hastened, but in none did the corneal reflex appear early in the sequence of metamorphic changes. Figure 3 shows a section of the forebrain with the implant.

Series B.—Agar pellets previously soaked in a 1:1,000 thyroxin solution were implanted in the fourth ventricles of seven tadpoles about 2 weeks prior to the expected onset of the reflex. Tactile stimuli were applied as in Series A. In three animals the reflex appeared overnight, and in the four others it appeared by the second day after implantation. This treatment also approximately halved the expected interval to f.l.e. Even so, in two of the cases, the reflex preceded f.l.e. by as much as 8 days. Such speedy appearance of the reflex suggests that the reflex mechanism was essentially mature at the time of operation, merely requiring thyroid activation to be set off.

Series C.—In this third series only agar implants were used. The tadpoles, all of which were in the later mid-larval stage, were divided into four groups. In Group I were placed ten untreated controls. In Group II there were four animals, each of which had had a thyroid-free agar pellet implanted in the fourth ventricle. This group provided a control to determine possible effects of the operative procedure. Four animals (Group III) received abdominal implants of agar pellets previously soaked in a 1:500 thy-

roxin solution. This group provided a control of the generalized metamorphosing effects of the implants. In twelve animals (Group IV) similarly soaked pellets were implanted in the fourth ventricle.

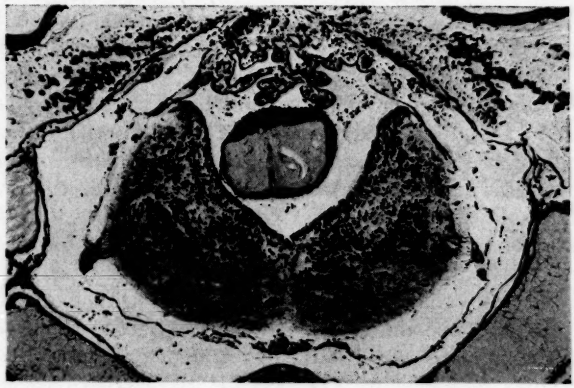

FIG. 2.—Thyroxin-soaked agar implant in the fourth ventricle. The implant has become encapsulated. Protargol-silver impregnation. ×41.

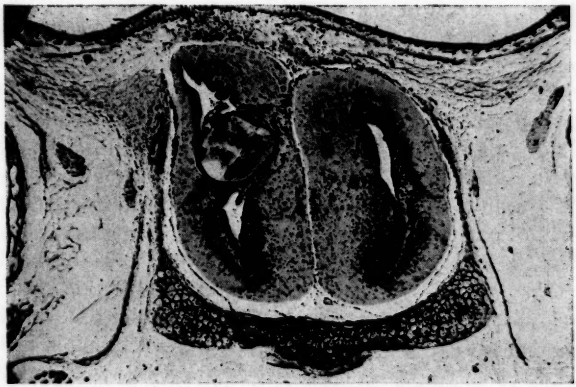

FIG. 3.—Thyroxin-soaked agar implant in the forebrain (right lateral ventricle). Ehrlich's haematoxylin-eosin. ×28.

In this series the appearance of the reflex was tested by strokes of fiber 4, in contrast to the previous series in which fiber 2 had been used. As stronger stimuli can be administered with fiber 4 than with fiber 2, it was to be expected that the reflex would be elicitable somewhat earlier than in the control group of Series A. On the contrary, it appeared later, preceding f.l.e. by from 1 to 4 days (average of 2.7 days) in the untreated controls (Group I) and by an average of 3.8 days in the operated controls (Group II). These results suggest that in the present stock of tadpoles the time differential between the onset of the reflex and f.l.e. is consistently less than in Series A.

Groups III and IV, which had received implants of thyroxin-soaked agar, developed at equal rates, each about twice as fast as normal. They displayed the usual features of intense thyroid stimulation, such as very rapid growth of the forelimbs and hind limbs, precocious appearance of the opercular perforation and emergence of the forelimbs, very rapid pigment changes, constriction of the body just behind the gills, and a disproportionate bulging of the eyeballs. Of the four animals with abdominal implants, one first displayed the reflex 6 days prior to f.l.e., while the others displayed it 1, 1, and 3 days after f.l.e. (Fig. 1, Bar VI). The aberrant behavior of the first animal is not readily explained.

In Group IV the implant could act locally upon the reflex center, as well as exert its general metamorphosing effects by way of the circulation. The appearance of the reflex preceded f.l.e. by from 1 to 11 days, or by an average of 5.2 days (Fig. 1, Bar VII). Because of the very great metamorphic acceleration of the body generally, the local effects of the implant were masked. Even so, the local action produced the 5-day lapse between the results shown in Bars VI and VII. If the single aberrant animal of Group VI were to be left out of consideration, the time lapse between the two groups would be 7 days.

DISCUSSION

In the anurans previously studied it had been impossible to elicit the corneal reflex prior to the period of metamorphic climax, despite the fact that both the afferent and the efferent portions of the reflex arc had been shown to be functional, or at least capable of functioning (Kollros, 1942*a*). This evidence suggested, first of all, that the failure of the reflex to be elicited in younger larvae might be ascribed to some deficiency of the reflex center in the larval hindbrain and, second, that this deficiency was normally overcome by the action of the metamorphosing agent, thyroid hormone. Attempts to bring about a premature maturation of the reflex mechanism through the use of thyroid hormone were made in two different ways: by thyroxin administration to the entire animal and by local implants of thyroxin-soaked agar pellets or rat thyroid tissue to the vicinity of the reflex center in the medulla oblongata. The addition of thyroxin to the water in which the tadpoles were kept advanced the metamorphosis of the whole animal markedly, but it failed to produce an appearance of the reflex earlier in the metamorphic sequence than was normal. In fact, the stronger the thyroxin concentration, the farther back in the sequence the onset of the reflex was pushed. Instead of first appearing 4 days before the emergence of the forelimbs from the branchial chamber (f.l.e.), as in the controls, the reflex first appeared from 2 days before to 4 days after f.l.e.

In contrast to the results of diffuse treatment, local application of thyroxin in high concentration to the tadpole hindbrain in the vicinity of the reflex center did produce an appearance of the reflex earlier in the metamorphic sequence than was normal. This was in addition to (i.e., superimposed upon) the advance in metamorphosis of the whole animal, which was presumably caused by the accumulation in the blood stream of some of the thyroxin from the implant.

Since, as has just been shown, acceleration of general metamorphosis through diffuse thyroxin treatment hastens the onset of the reflex less than it does other metamorphic changes, the nonlocalized effects of the thyroxin-agar implants could be expected to have the same result. Although the local effect of the im-

plant would advance the maturation of the reflex center, the diffuse effects would advance the metamorphosis of the rest of the body, thus decreasing the apparent influence of the implant upon the center, particularly since the center is less responsive to thyroxin than are other body structures. In diffuse treatment it was seen that the more concentrated the thyroxin solution, the greater was the disparity between the metamorphic advance of the reflex center and that of the rest of the body (Fig. 1, Bars I, II, III). A similar disparity was noted in the animals receiving the local implants. Those animals receiving pellets previously soaked in the 1:1,000 thyroxin solution showed a moderate metamorphic advance; but, because of the local influence of the implant, a still greater advance in the time of onset of the reflex was noted (onset of the reflex preceded f.l.e. by an average of 12.9 days). In those animals receiving implants previously soaked in the 1:500 thyroxin solution, the thyroxin content of the blood apparently became high enough so that body metamorphosis was greatly advanced, while the less responsive reflex center, although subjected to a still higher thyroxin concentration, did not have its metamorphosis advanced significantly more than did the body generally (onset of the reflex preceded f.l.e. by an average of 5 days). When compared to its control group (Ser. C, Group III), however, the advance is seen to be significant. Apparently the generalized effects of the implants mask their local influence upon the reflex center, at least in part. This is particularly true when the implants have been soaked in strong thyroxin solutions.

Because the experiments of Series A and C were carried out on two different stocks of tadpoles and because the sizes of the implants (and thus of the amount of thyroxin contained in the implant) varied, the figures quoted above may not be of the proper magnitude, although the differences between the results of Series A and C are probably significant. This in turn suggests that some still lower concentration of thyroxin might be found which, although capable of bringing the reflex center to functional maturity, would be too low to stimulate general metamorphosis appreciably. A period of more than 13 days would then be expected to intervene between the first appearance of the reflex and f.l.e.

The implantation of the thyroxin-agar pellets in the fourth ventricle in the vicinity of the center for the lid-closure reflex did not always induce a shift in the phase of metamorphosis during which the reflex first appeared. If the tadpoles were still so young at the time of implantation as to lack some of the digits of the hind limb, the first appearance of the reflex was premature only slightly as compared to that in untreated control animals. Apparently the reflex mechanism was not yet prepared to respond to thyroxin treatment. Tadpoles scarcely a week older than those just discussed, and receiving similar implants, do display the reflex prematurely (Ser. A). Apparently the reflex center has become so modified in that interval as to be able to mature under the influence of the thyroxin. In these animals the average length of the period between implantation and the first appearance of the reflex was about 12 days, while with diffuse thyroxin acceleration approximately 20 days would have been required. By using still older animals it was possible to elicit the reflex from 12 to 36 hours after implantation, whereas normally 7–12 days would have had to elapse. These results suggest that the development of the reflex mechanism to the

functional phase progresses in three stages: In the early larval and mid-larval stages the reflex mechanism is self-differentiating within the brain centers independent of hormone action (Weiss, 1941); in the later mid-larval period the center comes under the influence of thyroid hormone and through its action is brought to maturity; finally, when the metamorphic stage is reached, the hormone precipitates the setting-into-action of the reflex mechanism. As is suggested by Series B, the mechanism is ready for action in the premetamorphic period but would normally be delayed in its appearance until a few days before f.l.e. Local thyroxin administration, however, can terminate this waiting period.

SUMMARY

1. This study was carried out on the frog tadpole in order to determine whether or not the maturation of the center for the corneal reflex, located in the medulla oblongata, is under direct control of thyroid hormone.

2. Normally the reflex appears an average of 4 days prior to the emergence of the forelimbs.

3. Addition of thyroxin to the water in which the tadpoles are kept advances the first appearance of the reflex less than it does other metamorphic changes. The onset of the reflex precedes forelimb emergence by less than 4 days, or even follows forelimb emergence.

4. In contrast, the local application of the hormone in the mid-larval tadpole to the vicinity of the reflex center (implantation in the fourth ventricle of fresh rat thyroid fragments or of agar pellets previously soaked in thyroxin solutions) has the opposite effect. The onset of the reflex is advanced more than are other metamorphic events. In the older group of tadpoles of Series A this advance amounted to 8 days, on the average, with a period of 2 weeks required after implantation to bring the reflex mechanism to a functional state.

5. In those cases in which the implant comes to lie nearer one wall of the medulla than the other, the reflex first appears earlier on that side, presumably because of the greater thyroxin concentration.

6. Some of the hormone from the implants, circulating in the blood stream, accelerates general metamorphosis also; in the case of the strongest thyroxin solution (1:500) the general effect of the implant is so great as to mask the local effects. In such a series (C) the onset of the reflex occurred only 5 days prior to forelimb emergence. In thyroxin-treated controls whose metamorphosis has been accelerated to an equal degree the reflex usually appears at the time of forelimb emergence. The difference of 5 days between the two groups can be ascribed to local effects of the implant.

7. Implants of thyroxin-soaked agar pellets into the fourth ventricle of premetamorphic tadpoles results in the onset of the reflex in from 12 to 36 hours. This indicates that in these older animals the reflex center is essentially ready to function, requiring only the final stimulation provided by the hormone.

8. Similar implants made into young mid-larval tadpoles are ineffective in producing a premature appearance of the reflex, although they do speed up body metamorphosis for a time. Apparently the reflex mechanism in these younger tadpoles has not yet self-differentiated to the point where thyroxin can be effective in setting it into action.

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PHYSIOLOGICAL CHARACTERISTICS OF THE DIAPAUSE GRASSHOPPER EGG

II. CHANGES IN DENSITY AND WEIGHT DURING DEVELOPMENT*

(Four figures)

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DIAPAUSE in the grasshopper egg is a period characterized by morphological inactivity and a low level of metabolism. The preceding paper (Bodine and Robbie, 1940) presented experimental evidence to show that the stability of this inactive condition in eggs of the grasshopper *Melanoplus differentialis* diminishes as the diapause period progresses. During the latter part of the period it is possible to disturb the eggs enough, by alternate wetting and drying or mild centrifugation, to cause the embryos to resume active development.

* Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

Observations of the weight and density of eggs in the diapause state have been made for three successive years to see if any changes might be noted that could perhaps be correlated with this progressive decrease in stability.

Bodine (1929) measured the change in weight and water content of the grasshopper egg during development. Slifer (1938) records weight measurements that are quite similar to those in the present paper. Both of these investigators, however, show determinations for only the first few weeks of diapause. The present report gives figures for eggs that have been as much as a year in this condition. Johnson (1937) studied the absorption of water and the associated volume changes in the eggs of the capsid bug *Notostira* during embryonic development and found that the increase in wet weight and volume were directly proportional to the increase in amount of water.

Previous papers from this laboratory have described the normal development of *M. differentialis* eggs (Slifer, 1932; Boell, 1935), but for the convenience of the reader in interpreting the graphs below the following brief outline is presented.

At a constant temperature of 25° C. morphological development proceeds continuously for about 18 days after the eggs are laid. This is accompanied by a gradually increasing oxygen consumption, which also reaches a maximum at about the eighteenth day. At this time development slows down and finally ceases. The number of mitoses drops from 1,000–4,000 or more per embryo on the eighteenth to twentieth day to none after a week in diapause (Slifer, 1931). Respiration falls also, and by the thirtieth day it is only one-third that of the prediapause high (Boell, 1935). This inactive period following the twentieth day is known as "diapause" and may be of indefinite duration. A few eggs may resume development within 2 months, but others have been kept at 25° C. for over a year without starting to grow and yet have retained their viability.

If eggs which have been allowed to develop for 20 days or more at 25° C. are placed in refrigerators at 5°–10° C. and left for 2 months, the block to development is broken in most of them. When these eggs are then returned to 25° C., the embryos grow actively for 17 or 18 days and hatch. The oxygen uptake during this postdiapause period increases rapidly.

EXPERIMENTAL METHODS

All the eggs used in the experiments reported were kept under standard conditions. The egg pods were placed on a layer of damp sand in covered glass dishes shortly after they were deposited by the female grasshoppers. These dishes were kept at 25° C. in a covered tank in a thermo-regulated water bath. The points shown in the prediapause and diapause sections of Figures 2 and 3 represent determinations run on random samples chosen from these stock dishes. Postdiapause points were obtained by measuring several groups of eggs that had been placed at 5° C. after the twentieth day of development and left for 2 months or more. The zero-day postdiapause indicates the time at which the eggs were replaced at 25° C. and resumed their development.

The usual methods of determining density were too involved or slow to permit as many observations as were necessary in making a statistical study. Over twenty thousand eggs are represented by the points in Figure 2. To measure the density of this many individually was obviously impractical, and the method out-

lined below was improvised. This method is rapid and reproducible and gives sufficient precision to be within the range of variation of the biological material examined. The eggs were simply passed through a graduated series of sodium chloride solutions of increasing density, and the number that floated in each solution was recorded.

The solutions used were made up from a 20 per cent solution of ordinary table salt in distilled water in the proportions shown in Table 1. These were kept in covered glass dishes of about 500 cc. capacity. Density determinations were then made in the following manner. The eggs from several pods were washed in a 70 per cent alcohol solution and then in water, and the obviously injured or spoiled ones were discarded. One hundred of the remaining eggs were placed in a small basket made of fine copper screening which had been previously dipped in acid to remove the water-repellent coating. The basket with eggs was then transferred successively through the salt solutions, going each time into the solution of next greater density. Each time the container was kept under the surface long enough for any eggs that would float to come to the top. During the transfer from one dish to the next the basket was drained momentarily on a paper towel to prevent alteration of solution densities by the transfer of too much fluid from one to the other. After all the eggs had been floated out of the basket by the various solutions, the number in each dish was counted, and the eggs were removed by netting them up on another piece of screen.

For convenience in calculation the eggs were considered to be of the same density as the solution in which they first floated. Actually, only those which remained suspended were of the solution density, but the difference in density in the fluids in adjacent dishes in the series was small enough to make this assumption allowable and still secure sufficiently precise results.

TABLE 1

SALT SOLUTIONS USED FOR DENSITY MEASUREMENTS

Percentage of NaCl (by Weight)	Cubic Centimeters of 20 Per Cent (by Weight) NaCl Solution	Cubic Centimeters of H_2O	Measured Density	Percentage of NaCl (by Weight)	Cubic Centimeters of 20 Per Cent (by Weight) NaCl Solution	Cubic Centimeters of H_2O	Measured Density
8.....	160	240	1.065	13.....	260	140	1.100
9.....	180	220	1.072	14.....	280	120	1.108
10.....	200	200	1.078	15.....	300	100	1.116
11.....	220	180	1.086	16.....	320	80	1.124
12.....	240	160	1.094	17.....	340	60	1.129

To find the average density of a group of eggs, as plotted in Figure 2, the number of eggs in each dish at the end of the separation was multiplied by the density of the fluid in the dish. Then the sum of these products was divided by the total number of eggs. Distribution-density curves, as shown in Figure 4, *B*, may be drawn for groups of eggs by plotting the number of eggs floating in each dish against the density of the salt solution in the dish.

After these salt solutions are made up,

they may be used for a month or more if reasonable care is taken to prevent evaporation and the transfer of fluid from one dish to another during the determinations. Figure 1 shows the results of three measurements of the densities of a certain series of salt solutions—one at the time of preparation, another a week later, and the third 6 weeks later. Apparently there was some loss by evaporation, be-

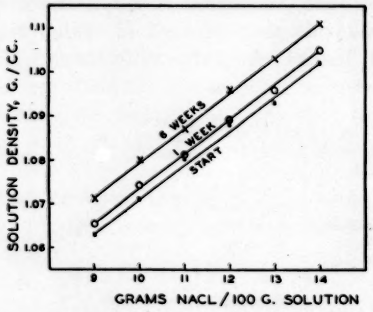

FIG. 1.—Change in density of salt solutions used in measuring density of grasshopper eggs. Abscissa: grams of sodium chloride in 100 gm. of solution, as prepared. Ordinate: solution density in grams per cubic centimeter as measured. Symbols: ■ = density at start; ○ = at end of 1 week; × = at end of 6 weeks.

cause the curves show that a definite increase in density occurred. But the fact that the lines through the points taken at the end of 1 week and at the end of 6 weeks are parallel to the original line indicates that no significant change in concentration arose from the passage of the eggs and their container from one solution to another.

If the salt solutions in the dishes had been standing for more than a week, the densities were measured again at the time the eggs were tested. This was done by weighing 10 cc. of each solution in a weighing bottle and computing the density from the formula

$$\text{Density} = \frac{\text{Mass}}{\text{Volume}}.$$

The maximum error involved in this method of determining density was less than 10 per cent of the density interval between one dish and the next one to it in the series. This technique of weighing was therefore sufficiently accurate for the purpose.

Alteration of water content within the eggs by osmotic action while the eggs were in the salt solutions would change the density and perhaps invalidate the results if it took place to a significant extent. The experiment represented in Table 2 was run to check this factor. Two hundred eggs were run through ten salt solutions in the manner previously described, and the numbers of eggs that floated in each dish were listed. Immediately after making this first determination and removing and washing the eggs, the eggs were passed through the solutions again. The second series of readings checked closely with the first, and the average density was almost identical. Then the eggs were allowed to stand for 20 minutes in the strongest salt solution

TABLE 2*

ALTERATION OF DENSITY OF EGGS IN CONTACT WITH A HYPERTONIC SODIUM CHLORIDE SOLUTION (17 PER CENT BY WEIGHT OF NaCl)

Percentage of NaCl in Solution	t_0	t_{repeat}	$t_{20\ min.}$	$t_{100\ min.}$
9.....	0	0	0	0
10.....	8	3	1	1
11.....	25	31	31	24
12.....	124	122	126	131
13.....	36	38	36	38
14.....	2	2	2	1
15.....	2	4	4	5
16.....	3	0	0	0
17.....	0	0	0	0
Average density.	1.0980	1.0982	1.0979	1.0990

* First column shows concentration of solutions used in measuring egg densities. Other columns list number of eggs that float in each solution. For further explanation see text.

of the series and determined again. After 100 minutes in this hypertonic solution a final density measurement was made. From Table 2 it may be seen that there is no change in the average density of the eggs even after they have stood for 20 minutes in a solution of a higher osmotic pressure than any of those needed for the measurement. Only a slight change in average density was apparent after 100 minutes. In the 100-minute trial it seemed that the lowest density eggs were those principally affected. Perhaps those that took in water more than the average and thus reduced their densities gave it up most readily in a hypertonic medium.

Weight measurements were made throughout on the same "chainomatic" balance. The eggs were always washed and dried on filter paper in the air for 20 or 30 minutes before placing them in a weighing bottle. After a 5-minute period of drying in the open air the eggs showed little further loss in weight. Four-day postdiapause eggs had lost only 4 per cent of their weight after 2½ hours of drying. Leaving the eggs exposed for 20 minutes before weighing appeared to be sufficient to remove the excess water from the outside of the shell and assure uniform precision in the measurements.

EXPERIMENTAL RESULTS AND DISCUSSION

The water content of actively growing and metabolizing cells is usually greater than that of cells in a resting or dormant state. Since the density and weight changes in the grasshopper egg are caused chiefly by the absorption of water, there may be some correlation between these changes and the metabolic condition of the egg. In making this correlation it is possible, on the basis of the data presented in Figures 2 and 3 and Table 3, to characterize four periods in the development of the egg.

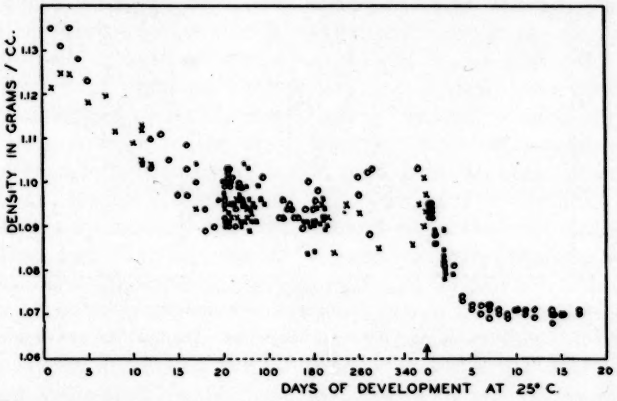

FIG. 2.—Density of *M. differentialis* eggs during development. Abscissa: days of development at 25° C. Arrow indicates start of postdiapause development. Ordinate: density in grams per cubic centimeter. Symbols: O = 1938 group of eggs; ■ = 1939 group; × = 1940 group.

During the prediapause period, from the time of laying to 20 days of development at 25° C., there is a decrease in density and an increase in weight.

During diapause density does not change appreciably, but there is an increase in weight. Table 3 shows a statistical analysis of the density and weight

determinations shown graphically in Figures 2 and 3. The 360 days of the diapause period represented were divided into three groups of 120 days each, and the mean and the standard deviation from the mean were calculated for each group. It is evident that the density change throughout diapause is insignificant. There is a definite increase in weight in the second period; but, from the figures given, it might seem as though there was a decrease from the second to the third. This apparent loss in weight is probably an artifact resulting from the small number of points in the third group and the lack of determinations at that age of the 1938 egg series which was responsible for a number of high points in the second group. Even without allowing for this it is apparent that an increase in egg weight does occur during diapause.

The third period which might be set off is that from the start to the eighth day of postdiapause development. Here the density decreases and the weight increases progressively, but both changes diminish and stop after the eighth day. The last period is that from the eighth day to the time of hatching at about the eighteenth day. During this time density and weight remain relatively constant.

In interpreting the observations listed

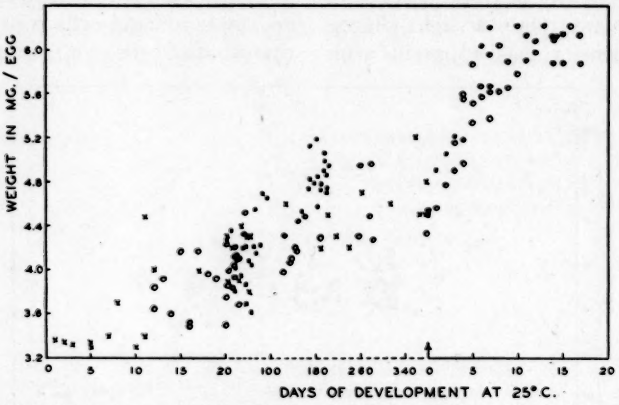

FIG. 3.—Weight of *M. differentialis* eggs during development. Abscissa: days of development at 25° C. Ordinate: weight per egg in milligrams. Symbols same as Fig. 2.

TABLE 3

RESULTS OF MEASUREMENTS OF THE WEIGHT AND DENSITY OF GRASSHOPPER EGGS DURING DIAPAUSE

	Age (Days)		
	20–140	140–260	260–380
Weight (milligrams per egg)	4.13±0.25	4.64±0.30	4.61±0.61
Number of eggs	4,600	2,700	1,400
Density (grams per cubic centimeter)	1.096±0.0039	1.092±0.0035	1.095±0.0069
Number of eggs	6,900	3,100	1,500

above, the following factors may be considered. During prediapause the embryo grows in size, the respiration increases, and the sensitivity of the respiration to 0.001 M cyanide is increased from about 0 per cent to 70 per cent (Robbie, Boell, and Bodine, 1938). These changes may be correlated with the increase in weight and decrease in density noted.

In diapause morphological development stops, respiration drops to a low level, and the sensitivity to cyanide is markedly diminished. It has been noted that the stability of the diapause block to development decreases with time and that eggs which have been in the diapause condition for a long time are more easily stimulated to resume growth than those which have only recently become inactive (Bodine and Robbie, 1940). This is quite similar to the phenomenon of dormancy in plant tissues (Meyer and Anderson, 1939). An increase in water content of the egg as it remains in diapause might indicate a changing physiological condition of the protoplasm that could be associated with the breaking of the developmental block.

Although the density during diapause seems to stay constant, there is still an increase in the water content of the egg. It may be seen from the equation

$$\text{Density} = \frac{\text{Mass of solids plus mass of liquids}}{\text{Volume}}$$

that, if volume increase is proportional to mass increase, the density will remain the same. Since the mass of solid matter within the egg does not increase, the proportionality between the total mass and volume cannot be kept constant when water is taken up. The density of the whole egg at the time of diapause is nearly enough like that of water, however, to make a density change difficult to notice unless the volume change is large. During diapause the eggs apparently take up enough water to show an observable weight increase but not enough to give a significant difference in density measurement.

After diapause has been broken, the rapid decrease in density and increase in weight which occur are again due to the uptake of water. If 0–2-day postdiapause eggs are left dry in the air and examined at the end of a week, it will be found that very few of them have developed. The drying has not injured them, because they will start to develop immediately if placed in a damp chamber. Slifer (1938) reports a similar stoppage of growth when the micropyle ends of the eggs are sealed so that water cannot enter. By the end of 7 or 8 days of normal development the egg has swelled so much that the elastic tension of the egg membranes is enough to prevent further expansion. This may be the explanation of the flatness of the weight and density curves during the last part of the postdiapause period.

Figure 4 illustrates another correlation between density and physiological activity. The points shown in Figure 2 are average densities of groups of one hundred or more eggs, but actually there is a considerable variation in a group which represents one day's laying of eggs by a number of different grasshoppers. The distribution of a mixed sample follows in general a bell-shaped curve, as in Figure 4, *B*. Figure 4, *A*, shows the oxygen consumption of fractions of various densities as indicated on the abscissa. The curves marked by × in both *A* and *B* are for eggs that have been in diapause for 55 days. Those indicated by an open circle are 280 days along in the diapause period. In both curves it may be noted

that the groups of eggs with the lowest densities have the highest respiration. Some of the highest points in the 280-day group may be abnormally high because of the presence of a few eggs that have spontaneously passed into the postdiapause physiological state but are still morphologically indistinguishable from the diapause individuals.

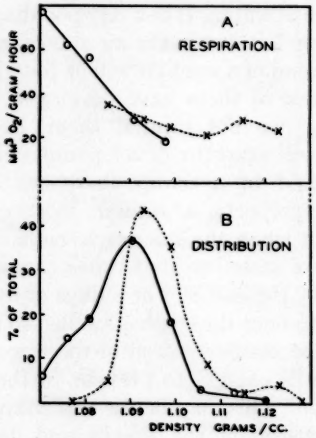

FIG. 4.—*A*, Respiration of fractions of two groups of eggs divided according to density. Abscissa: density of egg in grams per cubic centimeter. Ordinate: cubic millimeters of oxygen consumed per gram of egg per hour.

B, Distribution of eggs represented in *A* according to their densities. Abscissa: density of eggs in grams per cubic centimeter. Ordinate: percentage of total number of eggs in group.

Symbols: × = eggs in early diapause (55 days from laying); o = eggs in late diapause (280 days from laying).

A useful feature that comes out of this study of density is that of egg separation and sorting. If a uniform lot of eggs is desired for an experiment, it is much simpler and more reliable to sort them by means of density rather than to pick out the bad ones by gross examination only. This may be done by washing the eggs thoroughly (sterilizing them in 70 per cent alcohol helps in cleaning the surface), placing them in a sodium chloride solution in which they will float, and then, by progressive dilution and separation, discarding the heaviest and lightest eggs. The densest eggs will be found on examination to be those that are delayed in development or are spoiled in some way. The lightest eggs contain embryos that are more developed than the average. The intermediate group will usually be found to be remarkably homogeneous in respect to size and state of development.

SUMMARY

A rapid method for measuring the densities of large numbers of grasshopper eggs is reported, and a study of the accuracy of the method is given.

Constant values for weight determinations of eggs may be secured by allowing the eggs to dry in the air for 20 minutes before weighing.

The density of the egg decreases and the weight increases during the prediapause period.

A weight increase during diapause was observed, but the average change in density was not statistically significant.

Density decreases and weight increases for 8 days postdiapause. After that neither of these properties change appreciably.

The distribution of diapause eggs according to density follows a bell-shaped curve. The eggs of lowest density have the highest respiration.

It is possible to sort a group of eggs, by means of sodium chloride solutions, to give a fraction which is remarkably uniform in respect to size and stage of development.

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GRADIENT PATTERN IN DEVELOPING GILL, LEG, AND BALANCER OF TRITURUS

(Twelve figures)

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A PRECEDING study of differential dye reduction and reoxidation in earlier stages of *Triturus* development showed the existence of definite regional gradient patterns, characteristic for particular developmental stages and undergoing definite changes in the course of development (Child, 1943*b*). The present paper is concerned with patterns of developing gill, leg, and balancer, as indicated by differential intracellular reduction and reoxidation of methylene blue and Janus green and formation, reduction, and reoxidation of indophenol blue.

MATERIAL AND METHODS

Developing gills, legs, and balancers can be stained more rapidly by oxidized methylene blue and Janus green than early developmental stages; but, since reduced methylene blue penetrates more rapidly than the oxidized dye, minute amounts of sodium hydrosulphite can be used, as described in the earlier paper, both for rapid reduction of the dye in solution for the primary staining and for rapid oxygen decrease and intracellular reduction of methylene blue, Janus green, and also for reduction of indophenol blue after its intracellular formation. Less rapid oxygen decrease and reduction can be brought about by the oxygen uptake of the animals sealed in small volumes of dye solution or after the indophenol reaction in water or in very dilute indophenol reagents. These reductions, if gradual, are all differential and indicate the same gradient patterns.

Intracellular reoxidation of methylene blue after reduction, and partial reoxidation of Janus green from colorless to red give further evidence of the existence of gradient patterns, the progress of re-

oxidation being opposite in direction to that of reduction. After staining with low concentrations of methylene blue, either oxidized or reduced by hydrosulphite, developing gills and legs, unless very deeply stained, undergo partial or even complete decoloration in completely oxidized dye solution in shallow containers having large surfaces open to air. This is evidently a reduction, for more or less return of color results from bubbling air through the solution; but, when this is stopped, decoloration may occur again in a few minutes. The earlier stages of balancers show similar reductions. Since these reductions occur in the solutions in which staining took place, they suggest that intracellular methylene blue increases the ability of the cells to keep the dye reduced in the presence of sufficient oxygen to oxidize the dye in solution. With progressive intracellular accumulation of dye this ability to reduce decreases and disappears. Earlier developmental stages show essentially similar reductions but in lesser degree than gills and legs (Child, 1943*b*). Increase of respiration by methylene blue has been reported for various organisms. Concentrations of methylene blue from 1/10,000 to 1/50,000 and of Janus green 1/20,000 and 1/25,000 have been used on the stages concerned. Reduction and reoxidation of methylene blue can be repeated several times without evidence of injury; but, in some cases, after several repetitions of reduction, gradual atrophy of distal regions of developing gills and even of legs has been observed in animals kept for a week or more after experiment. Here there is differential injury without evident cytolysis.

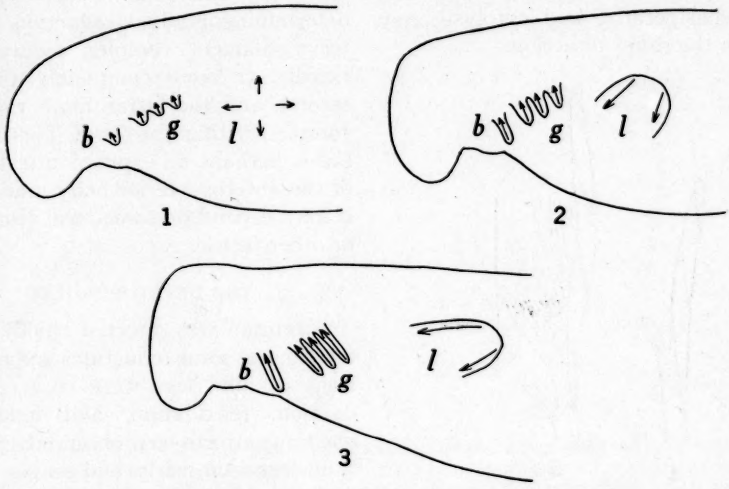
The indophenol blue reaction, reduction of intracellular indophenol with loss of color, and recoloration on reoxidation are all differential and indicate gradient patterns regionally identical with those of methylene blue and Janus green, although the indophenol reaction is associated with oxidase activity and the reduction of methylene blue and Janus green finally with dehydrogenase activity. It has been pointed out in earlier papers (Child, 1941, 1943*a*) that, although the indophenol reagents, para-aminodimethylaniline (dimethylparaphenylenediamine) and α-naphthol, are toxic in higher concentrations and that intracellular indophenol becomes toxic or lethal with progressive accumulation, the reaction will occur in very low concentrations of the reagents in living material without any evidence of toxic effect. One part in several million in water solution has been found sufficient to give the reaction in various organisms. In fact, it appears that the indophenol reaction is of little or no value as an indicator of spatial physiological patterns in development unless the reagents are used in concentrations low enough to give the reaction without marked toxic effect. The following concentrations of the reagents have been found satisfactory for developing gills, legs, and balancers of *Triturus:*

Aniline	α-Naphthol
1/240,000	1/300,000
1/128,000	1/160,000
3/320,000	3/400,000

With these concentrations the reaction becomes visible in the most rapidly reacting regions in from 5 to 15 minutes, and there is no evidence of toxic effect until the color becomes very deep. With one or more young animals in 1–2 ml. of fluid these concentrations may be considerably decreased before the reaction has proceeded far, and additions of 1–3 small drops (drop = 0.025 ml.) of the stock solutions (1/8,000 aniline, 1/10,000 α-naphthol) have often been found desir-

able for further progress of reaction. Differential decoloration by reduction of indophenol blue has usually been brought about rapidly by hydrosulphite, and reoxidation occurs gradually on stirring or bubbling air through the solution but is usually incomplete in the developing organs, as with methylene blue after reduction.

methylene blue and Janus green and differential indophenol reaction and reduction. The longer arrows indicate more rapid, shorter arrows less rapid, progress of the color changes. Successive arrows pointing in the same direction indicate continuation of progress in the same direction. Arrows oblique to the axis of the appendage indicate that the side from



FIGS. 1–3.—Three early developmental stages of balancer (*b*), gills (*g*), and foreleg (*l*), with gradient patterns, as shown by reduction and indophenol reaction, indicated by arrows.

If staining by methylene blue or Janus green or the indophenol reaction is carried too far, differential susceptibility to toxic action appears, reduction being most retarded in those regions which originally reduced most rapidly, with the result that the reduction-gradient pattern may be reversed or obliterated. Differential cytolysis may follow in the same direction as the progress of reduction preceding toxic effect.

As in the earlier paper on developmental pattern in *Triturus*, figures indicate diagrammatically, by means of arrows, the gradient patterns as they become visible in differential reduction of which the arrow points reduces or reacts earlier than the side toward which it points.

I am again deeply indebted to Dr. V. C. Twitty for his kindness in placing at my disposal developmental stages of *Triturus torosus*, *T. rivularis*, and *T. simulans*.

THE GILLS

In the three stages of outgrowth of the primary gill filaments in Figures 1–3, *g*, reduction of methylene blue and Janus green and indophenol reaction and reduction progress from the tips basipetally, as indicated by the arrows. After primary staining by methylene blue and

reduction the filaments, if not too deeply stained, remain so completely reduced in oxidized dye solution that, even to the naked eye, they stand out sharply as white against the stained body, and, after they are more or less reoxidized by increase of oxygen in solution, they usually reduce again rapidly. Toxic effect, indicated by deep color and retardation or absence of reduction, progresses from the tip basipetally, and cytolysis may follow in the same direction.

FIGS. 4 AND 5.—Two stages of gill development; arterial side with more rapid reduction on right.

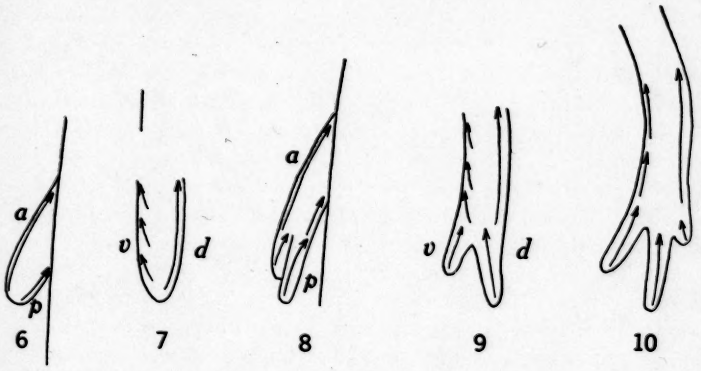
In later developmental stages of the filaments, after circulation has developed, the venous side of the filament is approximately anterior, the arterial side posterior or posteroventral, and the secondary filaments develop on the arterial side. Reduction and the indophenol reaction are basipetal in the secondary filaments, and the arterial side and the secondary filaments reduce more rapidly than the venous side (Figs. 4 and 5). This differential suggests that oxygen content of the cells may be lower on the arterial than on the venous side, in association with the lower oxygen content of the arterial blood. The gradient is slight in the short secondary filaments, but in the filament complex as a whole it is distinctly basipetal. Reoxidation progresses acropetally. In fully developed gills which have ceased to grow, the longitudinal gradient is apparently less strongly marked, reduction may sometimes be partly acropetal on the arterial side, and irregularities may appear among the secondary filaments. Under conditions determining gradual reduction the anterior filament complex reduces more rapidly or more completely than the second, and the latter more rapidly or completely than the third. This differential is perhaps an expression in the gills of the anteroposterior body gradient, or it may depend on some local circulatory or other factor.

THE DEVELOPING LEG

Attention was directed chiefly to the foreleg, but some reductions and reoxidations of hind legs were observed. Reduction, reoxidation, and indophenol reaction patterns were observed repeatedly in legs from early bud stages to legs with four digits and in all except early bud stages, in legs attached to the body and consequently with normal circulation, and in isolated legs without circulation. Reduction was brought about both by oxygen uptake of animals in sealed preparations and by hydrosulphite in open dye solution, without difference in results as regards gradient patterns.

As is well known, the foreleg makes its appearance as a slight rounded elevation on the side of the body. Even at this stage it is a region of somewhat more rapid reduction and indophenol reaction than surrounding regions (Fig 1, *l*). At a slightly later stage the bud appears more definitely outlined dorsally and posteriorly in consequence of dorsiposterior growth. At this stage reduction and in-

dophenol reaction progress from the dorsal or dorsiposterior border (Fig. 2, *l*), and on increase of oxygen after reduction of methylene blue or indophenol the bud remains largely or wholly reduced until toxic effects appear. Growth of the bud becomes increasingly posterior in direction, and reduction and indophenol reaction progress from the dorsiposterior or posterior tip (Fig. 3, *l*). At this stage or later, reduction and indophenol reaction the mesodermal gradient pattern seems to be the same, but under these conditions ectodermal reduction may be retarded and mesodermal differentials become less clearly visible. The outgrowing leg at this stage possesses a gradient pattern in three dimensions, as indicated both by reduction and by indophenol oxidase activity. The same pattern appears in later stages of leg development (Figs. 8 and 9). Reduction



FIGS. 6–10.—Developmental stages of foreleg with reduction and indophenol reaction gradient pattern indicated by arrows: *a*, anterior; *p*, posterior; *d*, dorsal; *v*, ventral. Longer arrows indicate more rapid, shorter arrows less rapid, progress of reduction and reaction. Figure 6 shows the dorsal, Figure 7 the lateral, outline of predigital stage. Figure 8 the dorsal, Figure 9 the lateral, outline of stage with two digits. Figure 10, dorsal outline of later stage in pronation.

tion are slightly more rapid on the dorsal than on the ventral side of the bud.

With further elongation of the leg the longitudinal gradient persists, and reduction and indophenol reaction are more rapid anteriorly than posteriorly and more rapid dorsally than ventrally (Figs. 6 and 7). Whether there is a difference between dorsal and anterior sides has not been determined with certainty, but the dorsal side has seemed to be somewhat in advance of the anterior in many legs. With slight staining or indophenol reaction mesoderm is not stained, and gradient pattern appears only in the ectoderm. After deeper staining or reand indophenol reaction progress proximally from the tips of the digits and more rapidly in the longer dorsal than in the shorter ventral digit; also, it can often be seen that the anterior side of the digits reduces more rapidly than the posterior.

As the foot develops and the walking position with palmar surface in contact with the substrate (pronation) becomes more characteristic, the primarily dorsal side of the leg becomes more posterior, the primarily ventral side more anterior. For example, in the leg outlined in Figure 10 in dorsal view as regards body of the animal, the primarily dorsal side of the foot is now posterior; and the third digit,

dorsal to the first in the primary orientation, is now posterior. The longitudinal reduction and indophenol reaction gradient from the tips of the digits proximally is present, as in earlier stages, with more rapid progress on the posterior (primarily dorsal) than on the anterior (primarily ventral) side below the elbow. Above the elbow the distal-proximal gradient is still visible, but other differences are apparently less than in earlier stages. With deep indophenol reaction localized areas of deeper color appear in the mesoderm, perhaps associated with skeletal development; these have not been given special attention.

From early stages of elongation on, reoxidation in the leg shows the gradient pattern in reverse; that is, regions of more rapid reduction and reaction show less rapid and usually less complete reoxidation than less rapidly reducing and reacting regions. In earlier stages (Figs. 6–9) the whole leg, or all except proximal levels, and in later stages the foot and more or less of the distal leg remain or rapidly become almost or entirely reduced in completely oxidized methylene blue solution after reduction. With further increase of oxygen by bubbling air through the solution, and often if the animal is made to swim actively for 10 or 15 seconds, more or less reoxidation occurs, with rapid reduction following cessation of bubbling or swimming. Here, as elsewhere, reduction of intracellular methylene blue in completely oxidized dye solution open to air suggests that the regions of more rapid reduction and greater indophenol oxidase activity also have or attain, in consequence of experimental procedure, so high an oxygen uptake that the free oxygen in their cells is not usually sufficient to permit oxidation of the dye.

Gradient patterns of the earliest stages of the hind legs have not been determined, but stages corresponding to Figures 6–9 show reduction and reoxidation patterns similar to those of the forelegs.

THE BALANCER

Reduction and indophenol reaction progress from the tip basipetally, and reoxidation is acropetal in the developing balancer (Figs. 1–3, *b*, and Fig. 11). In the fully developed balancer, reduction often occurs early at the tip, perhaps because of secretory activity, but usually progresses acropetally over the larger part of the length (Fig. 12). In other cases, however, little or no definite gradient pattern appears in the fully developed balancer, probably because it is a temporary organ approaching the end of its life.

REGENERATING GILL FILAMENTS, LEGS, AND BALANCERS

Regenerating tips of developing gill filaments after amputation do not differ greatly in reaction, reduction, and reoxidation from normal developing filaments. There is perhaps a little more rapid reduction and less reoxidation in the regenerating tissue, but developing gill filaments reduce so rapidly and reoxidize so little under the usual conditions that such differences are at best slight or uncertain. When a primary filament is amputated immediately distal to a secondary filament, the latter may substitute for the amputated part, and there is no regeneration.

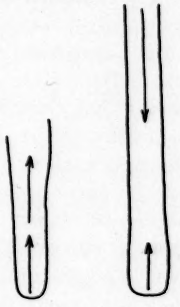
Reduction and reoxidation of methylene blue have been observed at various stages of regeneration following amputation at different levels of developing legs. The blastema reduces more rapidly and reoxidizes less rapidly and less completely than levels proximal to it, but in these respects it does not differ greatly from

the tip of the normally developing leg before appearance of digits or from the developing digits. Later stages of regeneration and intact developing legs have not been found to differ appreciably. Regenerating legs of adults have not been studied with the indicators; presumably, the earlier stages of regeneration in those would show greater difference from other parts than in the developing leg.

A few observations on regenerating balancers after removal of distal parts in developmental stages also indicated little or no appreciable difference in condition from intact developing organs.

NUCLEAR STAINING AND REDUCTION

Although aside from the chief purpose of this paper, it is perhaps of some interest that with moderately deep staining by methylene blue the ectodermal nuclei of gills, legs, and the whole body, and apparently all nuclei of the slender secondary gill filaments, stain in young animals without cytolysis or other evidence of death or injury of the cells and while the animals are normally active. Staining occurs either with oxidized dye or on reoxidation after penetration of dye reduced by hydrosulphite. It is not uniform and diffuse but shows nuclear structure. The stained nuclei are most clearly visible with partial reduction, either by hydrosulphite or by oxygen uptake of animals sealed in dye solution, because they reduce less rapidly than the cytoplasm in which they lie. At certain stages of reduction in legs, gills, or even on the general body surface, reduction is complete in the cytoplasm of a particular region while nuclei are distinctly stained but may reduce later. In legs, gills, and on the general body surface nuclear reduction shows the same general gradient pattern as cytoplasmic reduction, except when staining has been carried so far that more susceptible regions are injured or perhaps killed. Gills and legs in which stained nuclei have been visible do not necessarily lose their ability to reduce rapidly and may remain entirely intact. Stained internal nuclei have been seen in intact gill filaments and legs; it can be seen in the gills that blood corpuscles stain deep blue, and with partial reduction their nuclei become visible; the



FIGS. 11 AND 12.—Balancer gradient pattern. Figure 11, developing balancer. Figure 12, fully developed.

corpuscles can be repeatedly partially reduced and reoxidized if reduction is stopped while the heart is still beating. As regards other internal organs, no data are at hand.

Animals with nuclei of ectoderm and blood corpuscles and probably other nuclei of at least some internal organs stained may remain alive and intact on return to water, with rapid and often complete decoloration of gills and legs, though tips of gill filaments and digits may show some cytolysis. Blood corpuscles also undergo complete decoloration, and after several hours or overnight most or all nuclei of the dorsal body surface may be decolorized, while those of the ventral epidermis are mostly still stained. Both dorsal and ventral epi-

dermis may remain intact and evidently alive. These observations were made in part on *T. torosus*, in part on *T. rivularis*, and in various stages of gill and leg development after hatching, up to lengths of 12–15 mm.

DISCUSSION

In the earliest stages of leg development the primary gradient pattern is almost completely radial, becoming axiate in consequence of differential growth. Data regarding the earliest stages of gill filaments and balancer are lacking, but it seems evident that their axiate gradient patterns develop in the same way. In short, the longitudinal axis of gill filament, leg, and balancer appears as a new gradient pattern resulting from greater growth activity at or near the center of a localized area of activation with graded decrease in activity toward the periphery. This course of development from a primarily radial into an axiate pattern is very generally characteristic of buds and budlike outgrowths in both plants and animals, whether they develop into whole organisms or into organ systems. In the gills a new gradient pattern appears with origin of each secondary filament, and the circulation apparently becomes a factor in determining localization of these filaments and more rapid reduction on the posterior or posteroventral side of the primary filament and the filament complex.

The fact that the first secondary filament with its new gradient pattern appears at a rather definite distance from the tip of the primary filament and that other secondary filaments appear in definite order suggests a dominance of limited effective range in the primary filament. The possibility that lower oxygen content of the cells is a factor in determining development of secondary filaments on the arterial side of the primary filament is also suggested.

The anterodorsal-posteroventral gradient pattern of the developing leg appears to represent primarily persistence of the anterodorsal-posteroventral gradient pattern of the body from which the bud that becomes a leg develops. As the hydra bud, for example, shows evidence in its developmental pattern of the longitudinal gradient pattern of the hydra body from which it arises (Rulon and Child, 1937), so a small part of the gradient pattern of the body apparently becomes the anterodorsal-posteroventral gradient pattern of the amphibian leg. Again each digit involves appearance of a new polar gradient pattern and persistence in the digit of the anterodorsal-posteroventral pattern. If such gradient patterns constitute a physiological basis for differentiation, the gradient pattern of the developing leg provides a pattern in three dimensions. The gradient pattern of the balancer is longitudinal. In some developing balancers reduction appeared to be a little more rapid on the anterior than on the posterior side; but this differential was so slight, when visible, that its significance as a characteristic feature of balancer development has been regarded as doubtful. However, the posteroventral direction of growth and the circulatory pattern of the balancer suggest that the general gradient pattern of the body may play a part in its development. In any case, the developmental gradient pattern apparently does not persist very long in the fully developed balancer.

The existence of these gradient patterns in developing organ systems and the general gradient pattern present from early embryonic stages raise again the question whether such quantitative patterns can provide a basis for origin of

qualitative differences, that is, for localized physiological and morphological differentiation. Without attempting extended discussion, it seems desirable, in view of the data, to call attention again to a few points bearing on this question. The quantitative metabolic differences along a gradient unquestionably give rise to differences in concentration of various metabolites as well as of oxygen, carbon dioxide, and nutritive materials. It is a well-known fact that some reactions take place only when one or more of the reacting substances attain a certain concentration. Slight quantitative differences may determine activation or even formation of enzymes specifically different in action. It has been shown that slight differences in pH may determine combination of the same prosthetic group with specifically different proteins to form specifically different enzymes. Certainly, differences in pH must be present at different levels of gradient patterns, such as those present in amphibian development. In the light of what we know of metabolism at present, it is impossible to doubt that a great variety of quantitative differences must arise at different levels of an oxidation-reduction gradient in a system of such complexity as a protoplasm. If enzymes are important factors in the realization of genetic potentialities in development, it seems highly probable that such differences provide an entirely adequate basis for realization of different genetic potentialities at different gradient levels. In fact, it is highly probable that a quantitative gradient pattern does not remain entirely quantitative for any considerable time after its establishment, although quantitative factors may continue to be predominant for a longer or shorter time, perhaps throughout life, as they appear to be in many hydroids and planarians, or perhaps only until "definitive determination" of parts in development. The evidence from various forms of development shows beyond question that quantitative gradient patterns are generally characteristic of development, that they precede any distinguishable differentiation, that experimental determination of such patterns is followed by morphological differentiation in definite relation to them, and that when gradient pattern is obliterated axiate differentiation does not take place. And finally, if developmental pattern is not primarily quantitative, it is very difficult to account for occurrence of reconstitution of wholes from isolated parts without aid of something like Driesch's entelechy.

If these gradient patterns do constitute a physiological basis for localized differentiations, it is evident that within a certain range of quantitative variation of environmental factors relative, rather than absolute, differences along the gradient are chiefly concerned in determining the course of development. Within this range alterations of development are chiefly alterations of rate, though some effects on the course of differentiation may appear. In other words, the system exhibits a certain range of tolerance to environmental change. Beyond this range, however, modifications of development in certain definite directions appear and increase with increase in environmental change until certain parts are completely inhibited in development or parts not normally present appear. Since these modifications of earlier developmental stages do not show specific relation to particular environmental factors, the conclusion that they depend primarily on quantitative features of pattern appears justified.

Many lines of experiment show pro-

gressive increase in regional specificities in the course of development. So-called "definitive determination" of an organ or organ system evidently represents attainment of a certain degree of specificity not present earlier. What are the differences between a particular organ system and other parts preceding such determination if not predominantly or wholly quantitative? In terms of the gradient conception of developmental pattern the balancer, gill, and leg each represent in origin attainment of a certain degree of specificity in the system of activities and substrate originating at certain levels of the pattern of earlier stages. This, in turn, is the product of a pattern of still earlier stages with less definite regional specificities and so on, until we come to the primary quantitative pattern, the first step in axiate development. Probably we must go back to stages of oögenesis to find this primary pattern in eggs of certain animals; but in certain others—for example, certain hydroids, planarians, and annelids—quantitative characteristics of pattern persist throughout life; and, in spite of differentiations, re-establishment of the primary pattern is possible at all levels below hydranth or head, so that reconstitution of wholes from isolated parts is possible at all these levels.

Moreover, it is now a familiar fact that specificity of balancer, gill, and leg is not at first limited to the area directly concerned in their development but extends over at least a considerably larger area, a "field." The region of actual development of each of these organ systems under natural conditions is evidently the region of that field in which the particular activity representing initiation of balancer, gill, or leg development is most intense, so that development begins there earlier than elsewhere or is possible only above a certain threshold of intensity. If we activate another region of the field sufficiently, the organ system may develop there. What is the essential primary difference between the region of actual development and other regions of the field, if not quantitative?

And, finally, the pattern within each of these organ systems appears to be primarily a quantitative gradient pattern, differing specifically as a whole in some way from the gradient pattern of the whole animal and of the other organ systems. The urodele leg, for example, is capable of extensive reconstitution, and even development of a distal part from a proximal cut end may occur. In short, the leg in reconstitution is not very different from a hydroid or a planarian. Whatever regional specificities have appeared in development of the leg, reappearance of the primary pattern is still possible in spite of them.

The oxidation-reduction gradients described above show that quantitative gradient patterns are present from earliest stages on but give no information concerning origin or presence of regional specificities within these patterns. However, in the light of all the evidence from transplantation, explantation, and reconstitution, as well as the various lines of experiment directly demonstrating presence of gradient patterns, it appears impossible to conceive a spatial pattern consisting primarily of localized specific or qualitative elements which will provide an adequate basis for development in all its various forms and aspects.

SUMMARY

1. The presence of definite and characteristic oxidation-reduction gradient patterns in developing the gill, leg, and balancer of the urodele *Triturus* is indicated

by reduction and following reoxidation of intracellular methylene blue, Janus green, and indophenol and by intracellular formation of indophenol, all occurring in intact living organ-systems.

2. Except when differential toxic effects are involved, reduction and indophenol reaction show gradients in the same direction, and recoloration in reoxidation following reduction progresses in the reverse direction.

3. Reduction and indophenol reaction progress basipetally in outgrowing primary and secondary gill filaments and the later filament complex. The arterial side of the complex, on which the secondary filaments develop, reduces more rapidly than the venous side.

4. The virtually radial pattern of reduction and indophenol reaction in the earliest stages of leg development becomes an axiate pattern in consequence of differential growth. In the elongating leg, reduction and indophenol reaction progress from the tip proximally, more rapidly on the anterodorsal than on the posteroventral side; that is, a tridimensional gradient pattern is present in the developing leg, apparently resulting from the origin of a new longitudinal gradient and persistence in this lateral outgrowth of the anteroposterior and dorsiventral gradient pattern of the body. Each digit represents a new activation and a new longitudinal gradient.

5. In the developing balancer reduction and indophenol reaction progress from the tip proximally.

6. Gills, developing legs, and developing balancer reduce methylene blue in completely oxidized dye solution open to air.

7. Gradient patterns are not greatly altered in regenerating developmental stages of gill filaments, legs, and balancers.

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OXIDATION-REDUCTION PATTERN IN DEVELOPMENT OF A TELEOST

(Twenty-six figures)

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ONE or more of the following procedures—reduction and reoxidation of methylene blue and Janus green, the indophenol reaction in living material with very low concentrations of reagents, and reduction and reoxidation of intracellular indophenol—have shown the presence of definite spatial oxidation-reduction patterns in intact, living, and uninjured developmental stages of the chick (Rulon, 1935; Miller, 1941), echinoderms (Child, 1936*a*, *b*; 1941), *Triturus* (Child, 1942*b*, 1943*a*), and in developing ovaries of *Drosophila*

(Child, 1942*a*, 1943*b*). Some of the more important features of teleost pattern have also been briefly mentioned (Child, 1942*b*).

MATERIAL AND METHODS

The small teleost *Oryzias latipes*, originally from Japan, served as material. I am deeply indebted to Dr. D. M. Whitaker, of Stanford University, for permission to use embryonic material from his stock of this species. Four females, kept in the same aquarium with males, provided from ten to thirty eggs each morning with few exceptions. When eggs are extruded, long hairlike chorionic filaments remain in the female duct for a time, so that each lot is carried by the female for a few hours but can be freed easily without injury to the fish by one or repeated passings of a slender glass rod or tube, e.g., the tip of a dropper, from anterior to posterior on each side of the ventral surface of the swimming female.

Eggs are slightly more than a millimeter in diameter. The chorionic filaments often obscure embryonic regions but can easily be cut away. In consequence of high fluidity of the yolk, fragility of the embryo, and extremely hard and elastic chorion it was not possible to remove embryonic stages from the chorion intact. However, blastoderms and later stages were occasionally removed intact except for yolk; but removal is not necessary, since indophenol reagents, methylene blue, and Janus green all penetrate the chorion.

Methylene blue was used in concentrations of from 1:10,000 to 1:30,000; Janus green from 1:30,000 to 1:100,000. With these dyes the chorion stains rapidly and deeply, and with prolonged staining the embryo may be obscured. The indophenol reagents were used in concentrations so low that the reaction occurred gradually and with distinct differentials in living material without evidence of toxic effect. Stock solutions consisting of one small drop (40 drops per 1 ml.) of Eastman Company, 5 per cent solution of para-aminodimethylaniline (dimethylparaphenylenediamine) and 1 mg. α-naphthol, each in 10 ml. water or Ringer, were made daily, giving aniline 1:8,000 and α-naphthol 1:10,000. With the fish material 1–5 small drops of each of the stock solutions in 1 ml. of water or Ringer were used, giving approximate aniline concentrations of from 1:320,000 to 1:64,000 and α-naphthol from 1:400,000 to 1:80,000. Since the aniline stock solution gradually oxidizes and α-naphthol dissolves slowly, actual concentrations used were often less than these. However, the reaction occurs with one part in several million of the reagents, and within a wide range of low concentrations only time differences in appearance and progress of reaction and appearance of differential inhibition or cytolysis have been observed. Usually 2–3 small drops of each stock solution were used in 1 ml. water or Ringer.

Differential reduction of intracellular indophenol, methylene blue, and Janus green was brought about gradually by oxygen decrease through oxygen uptake of embryos sealed in small volumes of solution or rapidly by addition of minute amounts of sodium hydrosulphite in open solutions. Amounts of hydrosulphite used in this way are so small, and it oxidizes so rapidly, that weighing has not been attempted. With a little experience the proper amount can be very closely approximated. Hydrosulphite may be used in still smaller amounts merely to decrease oxygen in the medium somewhat before sealing, with further decrease and reduction by oxygen uptake of the embryos after sealing. In-

dophenol and methylene blue become colorless on reduction, and Janus green becomes red on partial reduction and colorless with further reduction. These well-known characteristics of the dyes are mentioned merely in order to call attention, first, to the fact that indophenol, like methylene blue, can be reduced by living tissues in low oxygen and, second, that loss of color in these dyes can be checked by the change of color of Janus green.

With the increase of oxygen on opening he sealed preparation, or by diffusion into the open solution after oxidation of the hydrosulphite, differential reoxidation and recoloration occur. Frequent change of position of the embryos by gravity in the sealed preparation and by stirring in open solutions provides for uniform distribution of indophenol reagents, dyes, and oxygen.

Indophenol provides three checks on pattern: differential intracellular formation of indophenol, differential reduction, and differential reoxidation. With methylene blue there are two checks: reduction and reoxidation. With Janus green the two stages of reduction—first to red, then to colorless—and reoxidation to red provide three checks.

In the absence of toxic effects the pattern of differential indophenol reaction and the pattern of reduction of indophenol, methylene blue, and Janus green are the same; that is, both reaction and reduction occur most rapidly in the same regions and progress in the same directions. Differential toxic effects may reverse or obliterate reduction and reoxidation gradients. In the following account the word "reaction" means the "intracellular formation of indophenol." Since conditions for reduction and reoxidation are essentially similar, except quantitatively, for all three indicators and since the reduction and reoxidation gradients with the three indicators are also similar, the terms "reduction" and "reoxidation" are used for any or all of the indicators without further specification, except in special cases.

Because of the deep staining of the chorion and relatively slow staining of the embryo by methylene blue and Janus green, indophenol reaction, reduction, and reoxidation were more extensively used than the other indicators; but results with indophenol were checked by reduction and reoxidation of the other two agents at the various stages, particularly where change of pattern was indicated. Except when on the microscope stage, embryos in indicator solutions were kept in darkness or dim diffuse daylight in order to avoid photodynamic effects. The data are based on several hundred embryos, and all features of pattern described have been observed repeatedly. Reduction and reoxidation have often been repeated several times on the same embryo until evidences of differential toxic effect appeared.

For observation in different positions the eggs were rolled about in the solutions by means of needles or by gravity in sealed preparations. The basal stumps of the chorionic filaments, like short spines, make it easy or extremely difficult to keep an egg in the desired position. Eggs supported on a few cotton fibers were sometimes more readily oriented and kept in position than on glass surface. No change in position of the embryo inside the chorion has been observed preceding active movement.

As in earlier papers on oxidation-reduction patterns, figures are diagrammatic outlines drawn from ocular micrometer measurements and sketches of stages of reaction, reduction, and reoxidation. Directions of progress, rather

than successive stages of reaction and reduction, are indicated by means of arrows, with areas of most rapid reaction and reduction lightly dotted in some cases. Longer arrows indicate relatively rapid, shorter, relatively slow, progress.

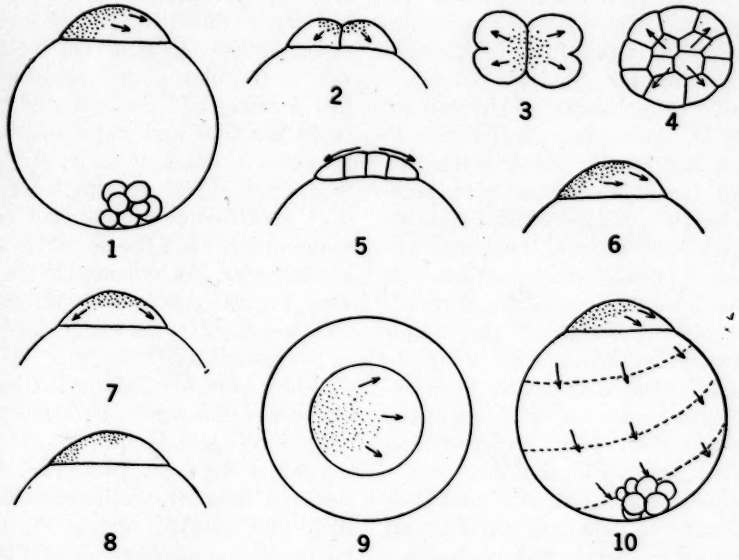
FERTILIZATION AND EARLY EVIDENCES OF AXIATE PATTERN

In the unfertilized egg many small oil droplets are uniformly distributed more or less peripherally in the transparent yolk, and there is no visible evidence of axiate pattern. As the blastodisk begins to form following fertilization, the oil droplets gradually move through the yolk away from the blastodisk and toward the opposite polar region. With frequent coalescence of droplets in the course of this migration, they aggregate first in a ring, which decreases in diameter with further coalescence of droplets until a few drops resulting from progressive coalescence and varying considerably in size and number are aggregated peripherally in a region almost, but not quite, opposite the blastodisk. In early gastrula stages, when the dorsal side can be identified with certainty, the oil drops are situated slightly ventral to the polar axis indicated by the blastoderm. In earlier stages, also, they are slightly on one side—probably, as will appear, on the ventral side, of that axis. Since there is no indication that they change position after migration during earlier stages, their migration and final position are probably expressions of a dorsiventral pattern of some sort in the yolk. It will be shown below that a polar-dorsiventral oxidation-reduction pattern corresponding in direction to the direction of migration of the oil droplets is present in the yolk. Figure 1 indicates the position of the aggregated drops in a blastoderm stage. The left side of the figure, with more rapid reaction and reduction in the blastoderm, indicated by stippling, is presumably dorsal.

PATTERN IN CLEAVAGE AND BLASTODERM STAGES

Indophenol reaction and staining are so slow in intact early stages that it has not been possible to obtain appreciable reaction or sufficient staining for reduction in the forming undivided blastodisk. In two-cell and four-cell stages the central regions of the blastoderm apparently show slightly more reaction than the margins, but the difference is at best very slight (Figs. 2 and 3). The central region seems to reduce more rapidly and reoxidize less rapidly than the margins; this differential, however, is also so slight that its existence has sometimes been doubted. In somewhat later cleavage stages, central cells are slightly smaller than peripheral; and the reaction, reduction, and reoxidation differentials become more distinct. They appear to be graded, rather than dependent on cell boundaries (Figs. 4 and 5). In these stages the indicators give no evidence of the radius or segment of the blastoderm in which the longitudinal axis of the embryonic area will appear. Slightly later in the many-celled blastoderm, about the time periblast appears, an oxidation-reduction pattern essentially similar to the amphibian dorsiventral pattern becomes distinct. This consists of more rapid reaction and reduction and less rapid reoxidation on one side and in the central region, with a gradation over the rest of the blastoderm. Using the aggregated oil drops as a means of orientation, the more rapidly reacting and reducing side is the future dorsal or embryonic side. The cells are apparently slightly smaller on this side than elsewhere. This pattern is indicated by stippling and arrows in the

surface view, Figure 1, and the optical section, Figure 6, both from the right side, according to position of the oil drops. In Figure 7 the pattern of the same embryo in a position at right angles to Figure 6 is indicated in optical section. The regional reduction pattern is similar, and reoxidation pattern is the reverse of the future embryonic side is a second region of increased oxidation-reduction, with the region between these two areas representing the lower levels of the two opposed gradients (Fig. 8). At this stage cytolysis, resulting from excessive indophenol, begins in the marginal area; but some cells in the central area often cyto-



FIGS. 1–10.—Pattern in cleavage and blastoderm stages. Fig. 1, blastoderm stage, presumably from right side, showing probable axial relations indicated by aggregation of oil drops slightly on one side, presumably ventral, of polar axis of blastoderm; more rapid reaction and reduction in one side, presumably dorsal, of blastoderm. Figs. 2–5, early cleavage stages with slightly more rapid reaction and reduction indicated in central region. Fig. 6, many celled blastoderm with more rapid reaction and reduction on one side, presumably the future dorsal side. Fig. 7, same blastoderm as Fig. 6 in optical section at right angle to Fig. 6. Fig. 8, slightly more rapid reaction and reduction in marginal and central than in intermediate region, as seen in earlier reaction and reduction stages. Fig. 9, pattern of blastoderm in surface view. Fig. 10, yolk reaction and reduction pattern with polar, and presumably dorsiventral, differential.

these, except when differential toxic effects are involved. In many blastoderms early reaction and reduction appear slightly more rapid in central and marginal parts of this blastodermic area than in the intervening region; that is, the primary central area of more rapid reaction and reduction is apparently still distinguishable, and the marginal region on lyze before those of the intervening region, suggesting that marginal and central regions are slightly more susceptible than the intermediate region. This pattern suggests that the marginal region of more rapid reaction and reduction is a region of progressive activation, primarily separate from the central region, like the dorsal lip of the amphibian embryo.

With further approach to gastrulation the central region becomes indistinguishable, and rate of reaction and reduction decrease slightly from the margin on the future embryonic side, as indicated in the apical surface view of the blastoderm, Figure 9.

It is a point of some interest that a definite oxidation-reduction pattern appears in the yolk at these stages and later. Probably a thin film of cytoplasm surrounds the yolk, but this pattern is not limited to the surface of the yolk region. It has been more clearly seen in reduction and reoxidation of methylene blue and Janus green than with indophenol, because the indophenol reaction in the yolk is slower and less deep than the staining, though the reaction does show the pattern. Reaction and reduction progress from beneath the blastoderm toward the oil drops, more rapidly on the side indicated as dorsal. Reoxidation progresses from the oil drops in the opposite direction. Reaction and reduction are less, and reoxidation is more rapid in the yolk than in the uninjured blastoderm, and the yolk still reduces and reoxidizes after the blastoderm has become incapable of reduction. Reaction and reduction pattern of blastoderm and yolk are indicated in surface view, presumably from the side, in Figure 10.

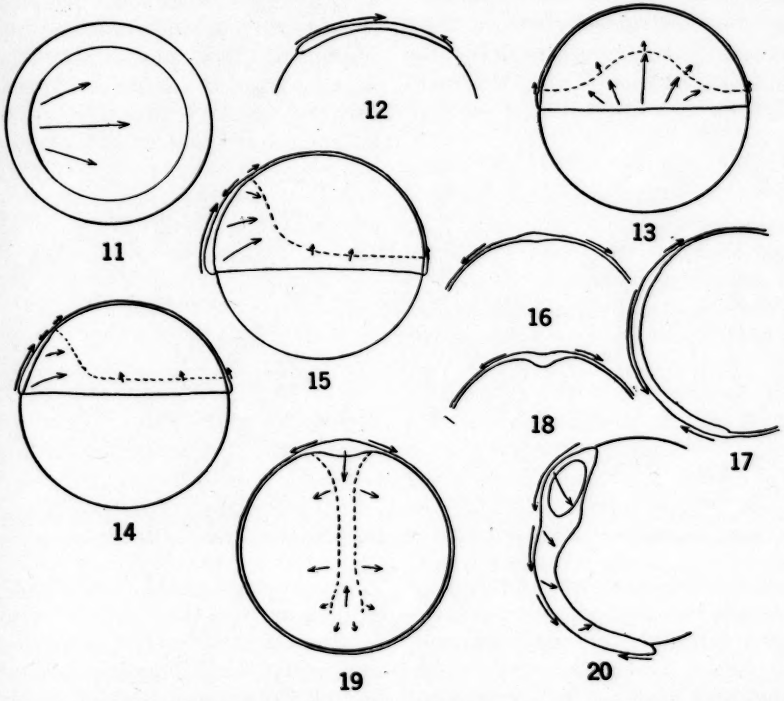
GASTRULA STAGES

The earliest stages of invagination are easily identified by the appearance of a more sharply defined boundary of the blastoderm on the embryonic side where invagination begins. This boundary is indicated in the surface view, Figure 11, by the heavier line at the left blastodermic margin. At this early stage of invagination, reaction and reduction progress toward the blastoderm center and somewhat laterally from this "dorsal lip," and the other half of the blastoderm reacts and reduces much more slowly and with little or no definite regional differential.

A slightly later stage of gastrulation is shown in optical section in Figure 12. Reaction and reduction are most rapid at the dorsal lip, with relatively rapid progress from the lip for a certain distance anteriorly, representing a very early stage of the embryonic area. On the opposite side of the blastoderm the germ ring is just forming and shows a slightly more rapid reaction and reduction than the blastoderm anterior to it. In general, "extraembryonic" regions react and reduce much more slowly and reoxidize more rapidly than the dorsal lip and embryonic area. As indicated in the surface view, Figure 13, essentially the same pattern is present in somewhat later stages of overgrowth of the yolk.

When overgrowth is about half-completed, a region of more rapid reaction and reduction begins to appear anteriorly in the embryonic area. In some embryos it seems to be faintly distinguishable at the stage of Figure 14, and at the stage of Figure 15 it is distinct. At this stage reaction and reduction are still most rapid in the dorsal lip, and the gradient extends anteriorly from the lip over most of the embryonic area, but a second gradient is now developing anteriorly. Reaction and reduction also progress laterally from the median region of the embryonic area (Figs. 13–16). In later stages of gastrulation, reaction and reduction become relatively and, so far as could be determined, absolutely more rapid in the anterior embryonic area, and the anterior gradient extends progressively farther posterior, obliterating and reversing more anterior levels of the posterior gradient. However, the posterior gradient persists in the posterior region even after

gastrulation is completed. The two opposed gradients at completion of gastrulation are indicated in optical section in Figure 17. Transverse optical sections at the middle or posterior third of embryos of this stage show a mediolateral and dorsiventral reaction and reduction pattern (Fig. 18) and the reverse reoxidation pattern.



FIGS. 11–20.—Beginning invagination, germ-ring, and later stages. Fig. 11, early invagination on dorsal side, indicated by heavier line bounding blastoderm at left; the "dorsal lip" the most rapidly reacting and reducing region of embryo. Fig. 12, longitudinal optical section of same stage as Fig. 11. Fig. 13, early embryonic area from dorsal side. Figs. 14 and 15, germ-ring stages from right side with anterior region of rapid reaction and reduction appearing. Fig. 16, transverse optical section in embryonic area of stage of Fig. 15. Fig. 17, median longitudinal optical section of embryo from right side at end of gastrulation. Fig. 18, transverse optical section in embryonic region of stage of Fig. 17. Fig. 19, reaction and reduction pattern in early stage of head development. Fig. 20, pattern of embryonic body.

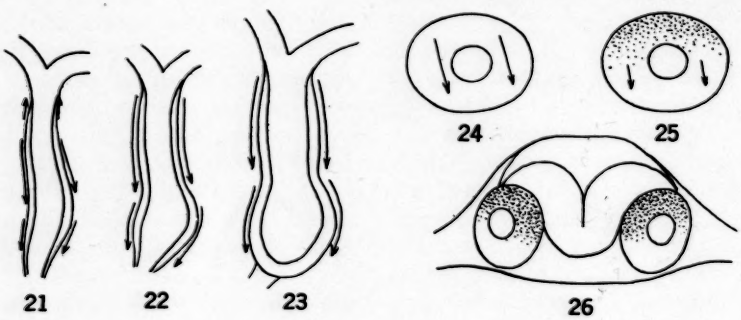
LATER STAGES

Reaction and reduction pattern of the developing embryonic body shortly after completion of gastrulation is indicated in surface view in Figure 19. The head region is now the most rapidly reacting and reducing region; the anterior gradient extends over most of the body length, with a short opposed gradient in the posterior region; and a mediolateral gradient is present over the whole length. In later embryonic stages there is little change in the general gradient pattern. The anterior gradient extends from the most rapidly reacting and reducing head region over most of the length, with marked de-

crease in rate of reaction and reduction in the somite region. The short gradient in the posterior region becomes more distinct as the tail develops, and is highly susceptible to toxic effects of indophenol and staining, so that its reduction is often retarded or prevented under conditions of reaction or staining not toxic elsewhere. The dorsiventral gradient, most conspicuous in the head region, is present at postcephalic levels. The general pattern in side view is indicated in Figure 20.

PATTERN IN THE EMBRYONIC HEART

When the heart begins to beat, it is a tube somewhat oblique to the longitudinal embryonic axis, connected at its anterior end with the vitelline veins and posteriorly with the arterial trunk. The earliest heart beat originates at a certain level of this tube at some distance posterior to the vitelline veins, and the contraction progresses posteriorly. The tube shows a reaction and reduction gradient with high end at, or probably slightly anterior to, the level at which the beat originates. Anterior to this level there is almost no reaction (Fig. 21). With further development the level of origin of beat and with it the high end of the gradient become more anterior until they are immediately posterior to the junction of the vitelline veins (Figs. 22 and 23). The ventricular region of the tube and later the ventricle show distinctly less rapid reaction and reduction than more anterior levels. In earlier stages of reaction color becomes distinct in the anterior region before the ventricular part shows any appreciable color. The toxicity of indophenol stops the heart before the color becomes deep; after the beat stops, reduction occurs, as in other parts of the embryo.



FIGS. 21–26.—Patterns of developing heart and eye. Figs. 21–23, three stages of heart development. Fig. 24, reaction and reduction pattern in eye. Figs. 25 and 26, pattern of pigment development in tapetum.

In stages after heart beat and circulation are well established, heart beat is weakened, retarded, and finally stopped by indophenol. Circulation stops while the heart still beats. After circulation stops, indophenol reduction begins almost at once without external oxygen decrease and progresses rapidly, or it may begin in the most rapidly reducing regions, even before complete stoppage of circulation. The same gradient pattern appears in these embryos as with external oxygen decrease. Evidently stoppage or sufficient retardation of circulation decreases oxygen supply to the tissues to the level permitting indophenol reduction. Probably oxygenation of the blood occurs chiefly in the vitelline circulation.

GRADIENT PATTERN IN DEVELOPMENT OF THE EYE

The optic vesicle is a lateral outgrowth from a region possessing an anteroposterior-dorsiventral gradient pattern. As might be expected, this gradient pattern persists in the eye and seems to become more distinct as its development progresses. In stages preceding invagination of the vesicle, reaction and reduction show a slight differential in progress from anterodorsal to posteroventral with reverse progress of reoxidation. As in the head, the gradient is chiefly dorsiventral, the anteroposterior component being slight (Fig. 20). In optic-cup stages preceding appearance of pigment in the tapetum the dorsiventral pattern is apparently more distinct than earlier (Fig. 24). If this apparent change is real, it may be associated with the increase in size of the optic cup.

Development of the black pigment in the tapetum provides another line of evidence showing the presence of a physiological gradient pattern in the optic cup. Pigment appears first in the dorsal region of the cup, and its formation is far advanced there before any pigment appears ventrally. Moreover, progress ventrally of pigment formation is slightly more rapid on the anterior, than on the posterior, side of the cup, so that the pigment gradient, like the oxidation-reduction gradient shown by the indicators, is from anterodorsal to posteroventral (Figs. 25 and 26). Since the pigment is a melanin and an oxidative reaction is concerned in its formation, this identity of reaction and reduction pattern and pigment pattern is not surprising. In the earlier stages of pigment formation, before it forms a continuous opaque layer dorsally and while the ventral region of the cup is still free from it, reaction, reduction, and reoxidation gradients are still visible. In later stages any pattern present is completely obscured by pigment.

No distinct difference in reaction and reduction between optic cup and earliest stages of lens development has been observed; but somewhat later, in what is probably the period of its greatest developmental activity, the lens appears to react and reduce slightly more rapidly than adjoining regions of the optic cup. After it attains full size, no definite difference has been seen.

REVERSALS OF GRADIENT PATTERNS

With increasing intracellular concentration indophenol finally becomes differentially toxic. Evidence of toxicity consists in retardation or absence of reduction and finally in cytolysis. Regions which normally show more rapid reaction and reduction show toxic effects earlier than others, and reversal in direction or obliteration of reduction and reoxidation gradients may result. Such reversal or obliteration of pattern is often seen after several repetitions of reduction and reoxidation on the same embryo. Differential toxic effects and reversal or obliteration of pattern have also been observed with Janus green. With methylene blue the chorion stains so deeply that the embryo becomes almost or quite invisible before evidence of differential toxic effect appears. Use of a considerable range of concentrations of indophenol reagents and dyes with different reaction and staining periods makes it possible to distinguish without difficulty normal patterns of reaction, reduction, and reoxidation from those due to differential toxic effects.

EFFECTS OF ISOLATION OF EMBRYONIC PARTS

Conclusions concerning embryonic pattern drawn from respiratory deter-

minations on isolated, and sometimes very small, fragments from different regions of embryos involve the assumption that respiration is not essentially altered by the isolations and the associated mechanical injury. In view of this situation it seemed desirable to determine whether the indicators would show any effects of isolation and injury on oxidation-reduction.

Stages preceding formation of the embryonic body and isolated parts of these stages removed from the chorion into water cytolyzed rapidly. In the most satisfactory of several Ringer modifications used they apparently remained in good condition only for several hours. Isolated parts of advanced enbryos are apparently less sensitive to the effects of the medium; but after heart beat and circulation are established, physiological condition is evidently altered by absence of circulation. As already noted, removal from the chorion of embryo and yolk intact was not possible. Occasionally blastoderms, early germ-ring stages, and embryonic bodies were removed intact, except for loss of yolk. In all such cases the lower sides of blastoderms and gastrulae and the ventral sides of later embryos, that is, the cells originally adjoining the yolk, showed much more rapid and intense indophenol reaction than other parts. In the embryo within the chorion with yolk intact these cells show the reaction last and react very slowly. After removal and loss of yolk they are, of course, more directly exposed; but under these conditions they react so rapidly and intensely that they become deeply colored in a few moments and before parts with undisturbed cells show any appreciable reaction. Moreover, if the reaction is allowed to continue more than a few moments before reduction, indophenol injury retards or prevents reduction, except under extreme conditions. In similar stages remaining within the chorion after loss of yolk by puncture and some pressure the same rapid and intense reaction occurs in this region. The evidence suggests an intense activation of these cells resulting from the altered conditions associated with removal of yolk.

In attempts at removal from the chorion all stages from blastoderms to advanced embryos are usually more or less injured, often torn apart, and blastoderms and early germ-ring stages break up readily into cell groups and single cells. All such isolated cell groups and cells show the indophenol reaction much more rapidly than even the directly exposed cell surfaces of intact undisturbed parts. Any local injury of a blastoderm, disturbing even a few cells, becomes the center of a region of much more rapid and intense reaction than in undisturbed parts. This effect is evident in decreasing degree for a short distance from the center of disturbance. Cuts and tears in the bodies of older embryos show much more rapid and intense reaction than intact parts with a decrement in the effect over a certain distance from the injured surface. Such a gradient in rate or intensity of reaction is visible for 0.2–0.3 mm. from the injury; evidently cells not directly injured are involved. In optic cups torn on the ventral side in attemps to remove embryos from the chorion the reaction may progress dorsipetally instead of ventripetally over the ventral part or even the whole cup.

Direct comparison of reduction in and about torn or cut regions and isolated cell groups or cells with reduction in intact regions of the same embryo in the same preparation usually has little significance because the injured region or part becomes deeply colored, while intact parts not affected by injury show only

slight traces of color superficially. Under these conditions the intact parts reduce first. However, with a longer reaction period for intact embryos than for injured or isolated parts of other embryos of the same stage, more or less comparable depths of color may be attained in both. In these cases, brought under identical conditions for reducing, reduction is usually much more rapid and reoxidation less rapid in the injured or isolated parts, cell groups, and cells than in intact embryos, even though the isolated parts are much smaller and with relatively much greater surface than the intact. With repeated reductions and reoxidations in the same material, reduction is usually retarded or fails to occur in the injured or isolated part earlier than in the intact embryos, evidently because of toxic effect.

These effects on indophenol reaction and reduction of mechanical injury and isolation of parts suggest that injury and isolation bring about a very considerable alteration in physiological condition, even in blastoderm stages. In the Ringer modifications used, the isolated parts did not live long enough to show whether this alteration is temporary.

DISCUSSION

The gradual aggregation of the previously uniformly distributed oil drops in a region slightly ventral to the polar axis of the blastoderm, in evident association with fertilization, indicates development or intensification in the yolk of a polar gradation of some sort with a slight dorsiventral component as regards the blastoderm. That a definite spatial pattern is present in the yolk is further indicated by the slight oxidation-reduction gradient in the same direction, with reoxidation progressing from the aggregated oil drops toward the blastoderm, somewhat more rapidly ventrally than dorsally.

As regards the presence of an oxidation-reduction pattern during development, the evidence is clear; but the question arises whether or to what extent the pattern really indicates intracellular physiological conditions which play an essential part in development rather than differences in permeability, in volume or thickness of tissues, or other incidental factors. As regards the reaction itself, the intracellular formation of indophenol, it has been found that in many forms—ciliates, coelenterates, echinoderm and ascidian embryonic stages, and developing *Drosophila* ovaries—the indophenol reagents penetrate almost immediately, even in very low concentrations, and that the reaction differential depends on conditions within the cells rather than on differences in permeability. In intact *Oryzias* material the indophenol reaction is less rapid than in many other forms; apparently the reagents do penetrate rather slowly, and regional differences in permeability are perhaps present; but, even if there is a permeability pattern corresponding to the reaction pattern, it is highly improbable that it is independent of physiological conditions within the cells. However, various lines of evidence make it sufficiently clear that the reaction pattern in *Oryzias* embryos is not primarily an expression of differences in permeability of cell surfaces.

First, the gradient patterns of indophenol reaction and reduction and reduction of methylene blue and Janus green are identical as regards axial relations. The reduction gradients evidently do not depend on differential penetration of reagents. In the absence of differential toxic effects regions of most rapid indophenol reaction are regions of most rapid reduction in low oxygen.

Second, indophenol reaction patterns and all reduction patterns preceding toxic effects show very definite relations to the course of development. Changes in these patterns are the earliest indications of regional changes in developmental activities and precede, rather than follow, the directly visible morphological changes. For example, the gradual development of more rapid reaction and reduction on that side of the blastoderm which will initiate invagination several hours later, and on which still later the embryonic area and longitudinal axis will develop, is evidence of change in physiological condition evidently concerned in bringing about the later morphological changes. Even if this pattern is present from the beginning of development without sufficient differential to be distinguishable by the indicators, the increase in distinctness preceding gastrulation indicates real physiological change significant for what follows. Development of the most rapid reaction and reduction in the region initiating invagination, the "dorsal lip," with a gradient extending anteriorly and somewhat laterally is obviously an expression of physiological developmental factors concerned in gastrulation and its results. Similarly, the appearance of a region of increased rate of reaction and reduction anteriorly in the embryonic area, with a gradient decreasing posteriorly, precedes head development and is evidently an expression of physiological factors concerned in that development.

Third, the association of the region of most rapid reaction and reduction in the developing heart with the region of origin of beat, the progress of both anteriorly along the tubular heart with progress of development, and the identity in direction of decrease in the reaction and reduction gradient and direction of progress of the contraction show clearly that the gradient pattern of the heart is an expression of physiological differences—in this case associated with function as well as with development.

Fourth, in the developing eye, reaction and reduction gradients and the gradient of pigment formation are identical and evidently represent persistence in the lateral outgrowth which becomes the eye of the general gradient pattern of the body. Also, the melanin pigment is a product of oxidative metabolism. These facts leave little doubt that these gradient patterns of the developing eye are expressions of physiological differences within the cells. Oxidation-reduction indicators have not been used on the urodele developing eye, but the pattern of pigment development is essentially similar to that in *Oryzias* (e.g., Barden, 1942). Regeneration of the lens from the dorsal iris margin is, in all probability, an expression of dorsiventral pattern similar to that in the teleost. A gradient in capacity to produce lens decreasing from the dorsal iris margin and some evidence that this might be altered in eyes transplanted in altered orientation was found by Sato (1930, 1933, 1935). The simplest interpretation of his and other data on urodele lens regeneration is in terms of gradient pattern.

Fourth, differential reduction may be entirely independent of differences in volume or thickness of tissues. It has recently been suggested by Philips (1942) that at least certain differences in rate of dye reduction observed by Rulon (1935) and Miller (1941) in the chick embryo are due merely to differences in thickness or volume of the respiring tissues. That is, in low oxygen the critical threshold for reduction would presumably be attained earlier in the greater volume or thickness of tissue, assuming that oxygen uptake

is the same in all parts concerned. Such differences in volume or thickness are undoubtedly factors in rate of reduction in some cases—for example, in *Drosophila* ovaries under certain conditions (Child, 1942*a*, p. 25, Figs. 19–21); but they are not involved in rate of indophenol reaction and not necessarily in rate of reduction. The indophenol reaction may be less rapid in the interior of solid cell masses than at the surface because the reaction in the superficial cells exhausts the reagents or decreases their concentration. Such cases, however, are usually readily recognized. When reduction gradients are identical with reaction gradients, volume or thickness of tissue is evidently not the determining factor. In the teleost and also in *Triturus* embryos reduction patterns show little or no relation to thickness of tissues. For example, the more rapidly reacting and reducing side of the teleost blastoderm is no thicker and no greater in volume than other parts. The rapidly reacting and reducing anterior region of the embryonic area in stages preceding head formation is the thinnest region of the embryonic area, and the dorsal lip is the thickest; but both reduce at about the same rate in earlier stages, and later the anterior, thinner region reduces more rapidly than the posterior thicker. After development of the embryonic body, reduction progresses ventripetally, although the ventral side is less directly exposed to the medium. If oxygen uptake were the same dorsally and ventrally, the ventral region might be expected to reduce before the dorsal, since it is presumably less able to obtain oxygen from the medium. The ventral side of the developing eye is more closely in contact with other tissues than the dorsal side, but reduction is more rapid dorsally.

Fifth, in general, recoloration gradients resulting from oxygen increase following reduction in low oxygen are opposite in direction to reaction and reduction gradients; that is, regions which react and reduce more rapidly show recoloration less rapidly. These reoxidation gradients seem to be associated with oxygen uptake, at least in many cases. With gradual oxygen increase in the medium, intracellular increase is presumably less rapid in regions of higher, than in those of lower, oxygen use. Differences in tissue volume or thickness may be factors in determining differences in some cases; but differential reoxidation, like reduction, is very generally independent of such differences in the teleost material. For example, the side of the blastoderm which reacts and reduces most rapidly shows recoloration most slowly, though it does not differ in thickness or volume from other parts. Later the thick dorsal lip and the thin anterior region both reoxidize much less rapidly than middle regions intermediate in thickness, and dorsal regions reoxidize less rapidly than ventral.

Sixth, reversals in direction of reduction and reoxidation gradients, resulting from differential toxic effects of intracellular indophenol or excessive staining by Janus green and less readily by methylene blue, evidently indicate alteration of physiological condition in the cells. In some cases the differential toxic effect may be just sufficient to obliterate gradient pattern. The differential modifications of development in various invertebrates and vertebrates by many chemical and physical external agents show how closely gradient patterns are associated with the course of development.

In the light of all the evidence there seems to be no question that the gradient patterns of indophenol reaction, reduction, and reoxidation, and reduction and

reoxidation of methylene blue and Janus green, as well as the patterns of differential susceptibility evident in the differential modifications of development, are all in close agreement in indicating presence of definite physiological patterns characteristic for developmental stage and undergoing definite changes in the course of development. Moreover, they are all evidently expressions, aspects, or correlated factors of one physiological pattern essential to development.

Comparison of reaction and reduction pattern in *Oryzias* with the differential susceptibility patterns of *Gadus*, *Tautogolabrus*, and *Fundulus*, as described by Hyman (1921), is of interest. The susceptibility pattern of *Fundulus* resembles the oxidation-reduction pattern of *Oryzias* more closely than do those of *Gadus* and *Tautogolabrus*. In a blastoderm stage of *Tautogolabrus* (Hyman, Fig. 10) the future dorsal lip is indicated as a region of high susceptibility but does not appear as such in figures of germ-ring stages. In *Gadus* the embryonic side of the germ ring is most susceptible (Hyman, Figs. 6, 7, 13, 14), but a posterior gradient is not shown until a late germ-ring stage (Hyman, Fig. 38). Hyman regards the species differences in susceptibility as associated with differences in the part played by the germ ring, as distinguished from the embryonic area in formation of the embryo. In absence of data on reaction and reduction in the forms studied by Hyman, the question whether these and the susceptibility patterns really differ remains open. However, the chief differences in the three susceptibility patterns, and between these and the oxidation-reduction pattern of *Oryzias*, are the developmental stages at which the dorsal lip and the anterior region of the embryo become evident in increased susceptibility or increased oxidation-reduction of indicators.

The similarity of the general oxidation-reduction pattern of *Oryzias* and the amphibian, *Triturus* (Child, 1943*a*) is striking and highly significant. In earlier stages the chief differences are the much greater reaction and reduction differential from the apical region basipetally and its persistence through gastrula stages In *Oryzias* cleavage stages and early blastoderm this differential is very slight, and no evidence of its presence has been found after beginning invagination The region of rapid reaction and reduction developing anteriorly in the embryonic area during gastrulation seems to be a new localization, presumably an effect of induction.

The increase in rate of reaction and reduction following isolation of pieces, cell groups, and cells and local disturbance of cell relations suggests that even in early stages the cells are sensitive to the change in conditions involved. Some evidence of similar effects of isolations and local injuries has been observed in amphibian embryos. Such changes may not involve change in rate of respiration; but, until it is certain that they do not, the conclusions of various investigators concerning developmental pattern which are based on determinations of oxygen uptake and CO_2 production on isolated fragments remain of uncertain significance. In an earlier paper (Child, 1943*a*) it was noted that, while certain of those determinations show no difference in aerobic respiration between the dorsal inductor and ventral regions of the amphibian embryo, there is a great difference in oxidation-reduction, as shown by indicators, and that the teleost oxidation-reduction pattern is essentially like the amphibian. The data suggest that oxidation-reduction indicators used in low concentrations on

living intact embryos may perhaps give more significant information concerning physiological developmental pattern than respiratory determinations on small isolated fragments. However, in this connection it should be noted that, according to Barth (1942), determinations of oxygen uptake of intact amphibian gastrulae and of gastrulae cut into six parts indicate that there may be a slight increase in rate due to cutting and a somewhat lower rate in the pieces than in intact gastrulae after twenty-four hours, but these differences may not be significant. Barth concludes that "cutting the gastrula into six parts has little effect on the rate of oxygen consumption."

SUMMARY

1. With very low concentrations of reagents, intracellular formation of indophenol, its reduction to the colorless form on decrease of oxygen content of the medium, its recoloration on reoxidation following reduction, and intracellular reduction and reoxidation of methylene blue and Janus green all show the presence of definite gradient patterns in living embryonic material of the teleost *O. latipes*. These patterns are characteristic for developmental stage and undergo definite changes in the course of development.

2. Except when toxic effects are concerned, gradient patterns of indophenol formation and reduction are identical. Recoloration on reoxidation progresses in the opposite direction. Differential toxic effects may reverse or obliterate reduction and reoxidation gradients.

3. In cleavage and early blastoderm stages reaction and reduction apparently progress from the central region to the periphery, with reoxidation gradient in the reverse direction; but the differential is very slight.

4. In the later blastoderm the future embryonic side on which invagination will begin reacts and reduces more rapidly than the rest; and with the beginning of invagination the invaginating margin, the "dorsal lip," becomes the most rapidly reacting and reducing region of the embryo.

5. This reaction and reduction gradient from the posterior end of the embryo persists throughout overgrowth of the yolk by the germ ring. When overgrowth is about half-completed, a second region of rapid reaction and reduction, with a gradient decreasing posteriorly, gradually appears anteriorly in the embryonic area, where the head will form. At this stage the longitudinal pattern consists of two opposed gradients. Reaction and reduction also decrease in rate from the median region of the embryonic area laterally and from the dorsal side ventrally in later gastrula stages.

6. In still later stages the anterior gradient becomes predominant and the posterior becomes shorter and less distinct, but a short posterior gradient characterizes the developing tail.

7. In its relation to the course of development the general pattern of *Oryzias*, as shown by the indicators, is essentially similar to the urodele pattern, as observed in *Triturus*.

8. The developing heart shows a reaction and reduction gradient with high end at or near the region of origin of beat; and, as this region progresses anteriorly in the developing heart, the region of most rapid reaction and reduction progresses with it.

9. In the developing eye, pigment formation in the tapetum progresses from the dorsal region ventripetally, somewhat more rapidly anteriorly than posteriorly; and reaction and reduction show the same gradient, that is, the lateral

outgrowth which becomes the eye retains the general gradient pattern of the body.

10. Reaction, reduction, and reoxidation in isolated pieces, cell groups, and cells suggest that, even in blastoderm stages, physiological condition may be considerably altered in such isolated parts.

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THE EFFECT OF 2, 4, 6-TRINITROPHENOL (PICRIC ACID) ON THE GROWTH OF THE CILIATE TETRAHYMENA GELEII[1]

(One figure)

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IN THE course of trials of various concentrations of picric acid for fixing cultures of *Tetrahymena* it was observed that a few of the organisms survived in concentrations as high as *ca.* 3×10^{-3} M. In somewhat lower concentrations the organisms grew quite satisfactorily, and the cultures passed through the usual phases. It was noticed that, as the cultures advanced in development, their color changed from the yellow of picric acid to red. Since ordinary cultures do not acquire this red color, it seemed reasonable to suppose that the picric acid was in some way taking part in the development of the culture. Since one possibility would be that it was stimulating the growth, a brief trial of this notion was carried out. It was found that, in twelve cultures to which a little picric acid had been added, the growth was significantly greater than it was in twelve similar cultures without picric acid. This led to further experimentation, the main results of which are presented in this paper.

[1] I am indebted to Dr. W. H. Johnson and to Dr. C. B. van Niel for advice and suggestions during the course of the work.

MATERIALS AND METHODS

The organism.—The organism which was used in this work is a strain of the holotrichous ciliate, *T. geleii*, which I obtained from Mr. J. O. Thomas. This organism has been in bacteria-free culture for several years and has been used for a number of experimental studies at Stanford University. Its morphology and taxonomy have been investigated by Furgason (1940).

The medium.—Media which have been used to cultivate this type of organism in bacteria-free culture have usually included yeast extract, proteose-peptone, tryptone (Difco products), or some similar nutrient material in a solution of 0.2–1 per cent. It has generally been customary to add to such a solution several salts constituting a balanced salt solution and, finally, to buffer the medium.

The last two items have been omitted in the experiments described here. The medium used throughout was a 1 per cent solution of Difco proteose-peptone in distilled water (twice glass-distilled). Such a solution has strong buffering properties and supports good growth of *Tetrahymena*. The addition of the salts of Osterhout's medium to the solution improves growth only slightly.

The solution was filtered in every case. Reeve Angel No. 280 paper was used. After filtering, the medium was measured in 10-cc. amounts into Pyrex culture tubes, 20 mm. × 150 mm., then cotton-stoppered and autoclaved.

Treatment with picric acid.—A sterile 0.01 M stock solution of 2, 4, 6-trinitrophenol was prepared by making the desired solution in distilled water and keeping it at 50° C. for 24 hours. Tests showed no evidence of contamination.

Sterile picric acid solutions of concentrations needed for the various experimental series were prepared by appropriate dilution of the 0.01 M picric acid solution into sterile distilled water. This operation, as well as all others involving the use of aseptic technique, was carried out in a small room built for the purpose, the atmosphere of which had been sprayed with propylene glycol.

Picric acid was added to tubes of sterile medium by introducing 1 ml. of the appropriate solution into each tube. One ml. of sterile distilled water was added to the control tubes. pH was checked with the glass electrode and was found to be the same in control tubes and in tubes treated with picric acid.

Inoculation.—In starting a given experimental series it was, of course, desirable to have a method of inoculation which would provide a fairly uniform inoculum for all the tubes of the series. This was achieved by means of a bulb pipette made from a 25-ml. volumetric pipette. The tube was heated just beow the bulb and drawn out to a diameter of *ca.* 1 mm. The fine tube extended about 5 inches below the bulb for convenience in withdrawing the contents of culture tubes. The upper end of the pipette was plugged with cotton, and the length and tightness of this cotton plug served as a method of limiting the rate of flow from the lower end of the pipette.

In inoculating, the pipette, containing about 10 ml. of the inoculum, was held perpendicular 1 inch or so away from the mouth of the tube to be inoculated. By the use of a mouth tube, air was drawn through the bulb of the pipette to stir the inoculum thoroughly, then the fluid was allowed to drop from the pipette by gravity. The first 2 drops were allowed to fall outside the tube, since bubbles were often contained in these drops. The pipette was then moved over the mouth of the tube, so that the third drop fell into the tube. In most series

only 1 drop was used to inoculate each tube.

In order to estimate the initial concentration of organisms in the experimental cultures, 3 or 4 drops of the inoculum were allowed to fall on a slide. These were dried, and the organisms were counted with a microscope. From these counts, the approximate initial concentrations were calculated.

Design of the several experimental series.—With some exceptions, eight tubes were used for each treatment or treatment combination in a series. After the tubes were prepared and labeled according to treatment, they were inoculated in random order.

In all series except Series XIII, the cultures were incubated for a selected period, usually 3 days, and at the end of that period were fixed for counting. The selection of the period of incubation was based on the idea of stopping growth when the fastest-growing cultures were near the end of the logarithmic phase. This type of experiment constitutes an extremely sensitive method of detecting differences in growth rate, if these occur in the logarithmic phase or earlier. A criticism frequently leveled at such experiments is that they provide no information except as to one somewhat arbitrarily selected point on the growth curve, and it must certainly be admitted that no rigorous deductions as to the shape of the curve can be made from this one point. The justification for this kind of experiment is that it provides an efficient and sensitive means of exploring a wide range of treatments or treatment combinations, many of which are not worthy of more than a tentative or preliminary trial. Results which are brought to light by this method may be tested, if desired, by series in which a number of points on the growth curve are determined.

The measurement data.—The organisms were fixed at the end of the incubation period by adding to each culture 1 ml. of a saturated solution of picric acid in normal hydrochloric acid.

Determinations of the population density of organisms in the cultures were made by means of a Sedgewick-Rafter counting cell and Whipple ocular micrometer. The method of using these was essentially the same as that described by Hall, Johnson, and Loefer (1935). Yields are given, for convenience, in numbers of organisms per 0.01 ml., disregarding the dilution caused by adding 1 ml. of fixative to the 11 ml. of the culture. Correction for this may be made in any particular case, if desired, by multiplying the yield value by 1.09.

Statistical analysis of the yield data.—The statistical problem presented by these data is mainly that of testing the significance of certain differences among the various mean yields of any particular series. Fisher's z test was used to find whether there was any significant differentiation throughout the series as a whole. If this proved to be the case, further testing of individual comparisons was done by means of "Student's" t test. The probability values derived from these tests are not given here. The P values for most of the comparisons discussed as significant in this paper are far smaller than 0.001, and none is greater than 0.01. Statisticians generally regard $P = 0.05$ as the arbitrary borderline value between significance and insignificance.

It is of interest to consider the standard errors of the general means of the several series. These varied from 1 per cent of the corresponding mean to 2.6 per cent, the average percentage being 1.7 per cent. This is reasonably low and should discourage criticism of the uni-

formity of the inoculation method, counting method, and other items contributing to the variation of the data.

Comparisons of these percentages with the numbers of the corresponding inocula indicate that inoculation is the chief contributor to total variation (Table 1). Precision is increased, up to a certain point, by using a larger inoculum.

TABLE 1

CORRELATION OF SIZE OF INOCULUM WITH DEGREE OF PRECISION OBTAINED

Series	Standard Error Given as Percentage of Mean	Inoculum No. of Organisms per Ml.
I	2.0	25
II	1.0	1,000
III	2.0	30
IV	1.2	100
V	2.0	30
VI	1.1	110
VII	1.7	60
VIII	2.6	28

EXPERIMENTS

For the sake of clarity in presentation, the experiments are divided into the three main groups into which they naturally fall, instead of being given in chronological order.

I. EFFCTS OF VARIOUS CONCENTRATIONS

Series I and II.—In these two series, each comprising 64 cultures, concentrations of picric acid from 6×10^{-8} M to 3×10^{-5} M were tested. The pH in both cases was 6.8. Series I was inoculated with about 25 organisms per milliliter and Series II with about 1,000 per milliliter. Both series were incubated at 25° C. Neither series showed any significant effect of picric acid.

Series III.—Concentrations of picric acid of 1.5×10^{-6} M to 1×10^{-4} M were tested. The pH of the medium was 7.0. The medium was autoclaved for 80 minutes at about 19 pounds pressure. The 64 tubes were inoculated with 1 drop each, from a 57-day-old culture of *Tetrahymena*. This gave about 30 organisms per milliliter as the initial count. They were incubated for 96 hours at cool room temperature in a well-lighted room. A concentration of 1×10^{-4} M picric acid gave significantly greater growth than the control, but the lower concentrations did not (Table 2).

TABLE 2

SERIES III

Molecular Concentrations of Picric Acid	Mean Yields in No. of Organisms per 0.01 Ml.
1×10^{-4}	306
5×10^{-5}	125
2.5×10^{-5}	94
1.2×10^{-5}	101
6×10^{-6}	115
3×10^{-6}	89
1.5×10^{-6}	89
Control	114

Series IV.—This series involved picric acid concentrations of about 4×10^{-5} M to 5×10^{-4} M. The medium was autoclaved 80 minutes at about 19 pounds pressure. The pH, checked colorimetrically, was 7.0 before and after autoclaving and also at the end of the incubation period.

Inoculations of 3 drops per tube from a 73-day-old culture of *Tetrahymena* were made. This gave an initial concentration of about 100 organisms per milliliter. The 64 tubes were incubated 67 hours at 25° C. It was hoped that the larger inoculum would result in an increase in precision, and apparently it did (Table 1).

Cultures with picric acid concentrations of 1×10^{-4} M to 5×10^{-4} M gave significantly greater growth than the controls, 2.2×10^{-4} M being the optimum concentration (Table 3). As in Series III, concentrations lower than 1×10^{-4} M gave no significant increase.

II. INFLUENCE OF VARIOUS CONDITIONS ON THE PICRIC ACID EFFECT

Series V.—This series was designed to compare the effects of picric acid under acid and alkaline conditions. Since 2, 4-dinitrophenol, a closely related compound, is known to be a respiratory stimulant, it was included in the series under the same conditions as was picric acid, in order to compare its effects on growth, if any, with those of picric acid. Both were used in a concentration of 1×10^{-4} M.

TABLE 3

SERIES IV

Molecular Concentrations of Picric Acid	Mean Yields in No. of Organisms per 0.01 Ml.
5×10^{-4}	303
3.3×10^{-4}	313
2.2×10^{-4}	332
1.5×10^{-4}	310
1×10^{-4}	299
6.6×10^{-5}	261
4.4×10^{-5}	254
Control	245

The medium was divided into two parts, one adjusted with hydrochloric acid to pH 6.0, the other with sodium hydroxide to pH 7.8. These were dispensed into tubes and autoclaved about 20 minutes at 15 pounds. The series consisted of 18 sets of the six treatments (Table 4)—108 cultures in all. They were incubated for 3 days at room temperature.

Dinitrophenol was toxic at the lower pH and had no significant stimulating effect at pH 7.8.

The picric acid stimulating effect was the same under both conditions (Table 4).

Growth was distinctly poorer in the alkaline cultures than it was in the acid cultures.

Series VI.—This series was planned with the idea of comparing picric acid effects at neutrality with those under alkaline conditions. The media were adjusted to pH 7.0 and 7.9. Instead of the usual 20 minutes at 15 pounds, the tubes were autoclaved for 45 minutes at 19 pounds, since there are some microörganisms which may survive the shorter period if the medium is alkaline. Thought was not given at the time to the possibility that the long autoclaving would hydrolyze, or in some way markedly alter, the complex components of the medium, particularly at the higher pH.

TABLE 4

SERIES V

pH	Treatment of Cultures	Mean Yields in No. of Organisms per 0.01 Ml.
6.0	Control	164
6.0	1×10^{-4} picric acid	212
6.0	1×10^{-4} dinitrophenol	0
7.8	Control	116
7.8	1×10^{-4} picric acid	155
7.8	1×10^{-4} dinitrophenol	124

The 64 tubes were inoculated with about 110 organisms per milliliter from a 22-day-old culture of *Tetrahymena*. They were incubated at room temperature (22.5°–26° C.) in diffuse daylight for 3 days.

The effect of the picric acid (1×10^{-4} M) was to increase the growth in the neutral cultures and to inhibit it somewhat in the alkaline cultures (Table 5).

The medium at pH 7.9 supported better growth than that at neutrality, presumably because of the hydrolyzing effect of autoclaving at the higher pH.

Series VII.—This experiment was for the purpose of comparing the effects of long autoclaving (involving hydrolysis and perhaps other changes) with those of the usual short sterilization time. This was carried out at pH 7.0 and 5.0. Alkaline media were not used, because the short sterilization time might have been

inadequate. After pH adjustment, part of each set of media was sterilized for 20 minutes at 15 pounds, and the rest for 80 minutes at 19 pounds. The pH was tested after the 20-minute autoclaving and was found to be unchanged.

The 32 tubes comprising the series were inoculated with about 60 organisms per milliliter of a 40-day culture of *Tetrahymena*. They were incubated in darkness at *ca.* 25° C.

TABLE 5

SERIES VI

pH	Treatment of Cultures*	Mean Yields in No. of Organisms per 0.01 Ml.
7.0	1×10^{-4} M picric acid	517
7.0	Control	404
7.9	1×10^{-4} M picric acid	550
7.9	Control	622

*The medium was both autoclaved and used at the pH given.

Picric acid (1×10^{-4} M) stimulation occurred in all the combinations except that of pH 5.0 with 80-minute autoclaving, in which case the net growth was much greater, but was inhibited by picric acid (Table 6). It is significant that this is the only combination in which marked hydrolysis would be expected to take place.

pH made no difference in either net growth or picric acid stimulating effect in the 20-minute series.

Series VIII.—In order to separate the effects of pH and hydrolysis, which are, of course, somewhat confounded in Series VII, an experiment was needed in which hydrolyzed medium was tested against unhydrolyzed medium, both at pH 7.0. For pH 5.0 this comparison is available in Series VII, where the autoclaving had such a marked effect.

For the present series the medium was adjusted to pH 5.0. Half of it was set aside, and the other half was autoclaved 60 minutes at 19 pounds pressure to hydolyze. Both halves were then adjusted to pH 7.0, tubed, and autoclaved 20 minutes at 15 pounds.

Picric acid was used in a concentration of 2×10^{-4} M.

The tubes were inoculated with about 28 organisms per milliliter from a 69-day-old culture of *Tetrahymena*. They were incubated at 24° C. for 75 hours in a water bath. Since it was desired to know if exposure to light would influence the results, 32 of the tubes were inside a light-tight box and 32 were outside, exposed to strong artificial light. Effective circulation of water was maintained through the box, and frequent checks of the water temperature inside and outside of the box gave identical readings.

TABLE 6

SERIES VII

pH at Which Medium Was Autoclaved and Used	Time of Autoclaving (In Minutes)	Treatment with Picric Acid	Mean Yields in No. of Organisms per 0.01 Ml.
7.0	20	1×10^{-4} M	366
7.0	20	Control	260
7.0	80	1×10^{-4} M	303
7.0	80	Control	168
5.0	20	1×10^{-4} M	349
5.0	20	Control	239
5.0	80	1×10^{-4} M	524
5.0	80	Control	806

A statement of the results of this series is simplified by the two facts that in every pair of combinations picric acid has a significant stimulating effect and that the combinations untreated with picric acid do not differ significantly among themselves (Table 7). One may therefore say at once that the secondary factors (hydrolysis treatment and light) do not have any significant effect in this series except in connection with the picric acid effect.

Picric acid stimulation, present throughout, was much greater in hydrolyzed medium than in unhydrolyzed medium. In the latter medium, however, the picric acid stimulating effect was significantly augmented by light.

Series IX.—It had been pointed out that the effects of dinitrophenol (on respiration) are often limited to narrow ranges of concentration or pH, so it was thought worth while to try it on growth under conditions different from those of Series V. As a preliminary, it was compared with picric acid, both in a concentration of 2×10^{-4} M, in medium which had been adjusted to pH 5.0, autoclaved 60 minutes at 19 pounds, and readjusted to pH 7.0 before final sterilization. The 24 tubes, including 8 controls, were inoculated with about 7 organisms per milliliter of a 2-month-old culture of *Tetrahymena* and incubated at 24.5° C. for 108 hours.

TABLE 7

SERIES VIII

Treatment of Medium and Cultures	Mean Yields in No. of Organisms per 0.01 Ml.
Light, hydrolyzed, 2×10^{-4} M picric acid	477
Light, hydrolyzed, control	49
Light, not hydrolyzed, 2×10^{-4} M picric acid	223
Light, not hydrolyzed, control	66
Darkness, hydrolyzed, 2×10^{-4} M picric acid	445
Darkness, hydrolyzed, control	63
Darkness, not hydrolyzed, 2×10^{-4} M picric acid	128
Darkness, not hydrolyzed, control	67

The usual marked stimulating effect of picric acid was present, but dinitrophenol was completely inhibiting. The dinitrophenol-treated tubes were later reinoculated from a culture in the logarithmic phase, but the result was the same.

Series X.—Medium prepared as in Series IX was used for this series also, except that the pH was readjusted to 6.4 instead of 7.0. Dinotrophenol concentrations of 7.5×10^{-7} M to 1×10^{-4} M were tried. Included in the series as controls were tubes treated with 2×10^{-4} M picric acid, as well as tubes of the plain medium. The series was inoculated with about 16 organisms per milliliter from a culture in the logarithmic phase. Dinitrophenol inhibiting effects became distinct at a concentration of 1.25×10^{-5} M. The effect was progressively greater at higher concentrations, and at 1×10^{-4} M no growth occurred. Picric acid, 2×10^{-4} M, had a small but definite inhibiting effect, about like that for 1.25×10^{-5} M dinitrophenol.

Series XI.—It is known that the rate of O_2 penetration into a culture of protozoa may limit the growth rate in early stages. Furthermore, tube cultures such as those described are not efficiently aerated. In view of these facts, it seemed that picric acid—a potential oxidizing agent—might have its stimulating effect by counteracting the lack of O_2. Accordingly, the effects in tubes were compared with those in 250-ml. flasks containing 10 cc. of medium (Table 8). Picric acid was used in a concentration of 2×10^{-4} M, and dinitrophenol 1×10^{-5} M was included in the series. The medium was prepared as in Series IX and readjusted to pH 7.0. The flasks and tubes were inoculated with about 7 per milliliter from a 2-month-old culture and incubated at 24.5° C. for 90 hours. The flasks were not shaken. In an earlier attempt, gentle shaking had been used during incubation, with the result that most of the flasks failed to develop at all.

From the present series it is clear that growth was not so rapid in tubes as in flasks, indicating that O_2 penetration was a limiting factor.

Picric acid had a marked stimulating effect in both tubes and flasks, but the effect was more pronounced in tubes.

A concentration of 1×10^{-5} M dinitrophenol had a stimulating effect, which, though distinct, was much less than the picric acid effect. Further, the effect was about the same in flasks as in tubes.

Series XII.—There is abundant indication in the series so far described that

TABLE 8

SERIES XI

Treatment of Cultures	Mean Yields in No. of Organisms per 0.01 Ml., Corrected for Evaporation
Flask, control	85
Flask, 2×10^{-4} M picric acid	307
Flask, 1×10^{-5} M dinitrophenol	123
Tube, control	38
Tube, 2×10^{-4} M picric acid	279
Tube, 1×10^{-5} M dinitrophenol	61

the stimulating effect of picric acid is associated in some way with the nutrient medium used. It seemed that tests of the respiration might throw some light on this, as well as on the question of whether picric acid would stimulate respiration.

For this purpose conventional Warburg vessels were used, in a water bath at 25° C. The organisms were reared in 1 per cent proteose-peptone, then concentrated and washed in Osterhout's medium, buffered at pH 7.0 with phosphate buffer. During the run, proteose peptone (prepared as in Ser. IX) was added to bring the concentration to 0.1 per cent. Picric acid was added to make 2×10^{-4} M. Two control vessels were used.

While no dependable conclusions can be drawn from the results of this one trial, the indications were that picric acid did not increase the respiratory rate of the organisms in balanced medium. The addition of the proteose-peptone increased the rate; but, when picric acid was added with the nutrient, the increase in rate was greater.

III. THE GROWTH-TIME RELATIONSHIP

Series XIII.—Two very important points which cannot be definitely settled by series of the type just given are the questions of where, in the growth curve, the stimulating effect takes place and whether the effect results in a difference in maximum populations. The answers to these questions are of the greatest importance in attempting to reach a conclusion as to the reason for the effect in question. This series, in which cultures were fixed at 12-hour intervals for 220 hours, was designed to answer these questions.

The medium used was adjusted to pH 5.0, autoclaved 60 minutes at 19 pounds, and readjusted to pH 7.0 before final sterilization. Picric acid was added to half of the tubes to give a concentration of 2×10^{-4} M. The tubes were inoculated from a 3-month-old culture of *Tetrahymena*. They were incubated at 24.5° C. Because of the initial sparsity of organisms, the first set of tubes was not fixed for counting until the fortieth hour. Each point in the curves is the mean of the yields of four cultures (Fig. 1). The shape of the curves is similar to that obtained by Phelps (1935) with the same or a closely related organism.

The slopes of the logarithmic parts of the two curves were calculated from the data, using for the picric acid curve the first four points (sixteen measurements) and for the control curve, the first seven points (twenty-eight measurements). The significance of the difference between the two slopes was tested by the t test, which gave $P = 0.01$.

Fission rates calculated from the

slopes are *ca.* 1.7 divisions per day for control cultures and 2.7 divisions per day (about a 60 per cent increase) for the cultures containing picric acid.

It can thus be confidently stated that (1) picric acid causes a stimulating effect on the growth of *Tetrahymena* in the logarithmic phase, and (2) it does not, in this series, cause any difference in the maximum population of the cultures.

medium was readjusted to pH 7.0, no improvement of the growth properties by the hydrolyzing treatment were in evidence. The pH itself seemed to have no marked effect on growth in unhydrolyzed medium, but in hydrolyzed medium, a nonneutral reaction gave (*a*) a net increase in growth and (*b*) an inhibiting, instead of a stimulating, effect of picric acid. Medium which has been hydro-

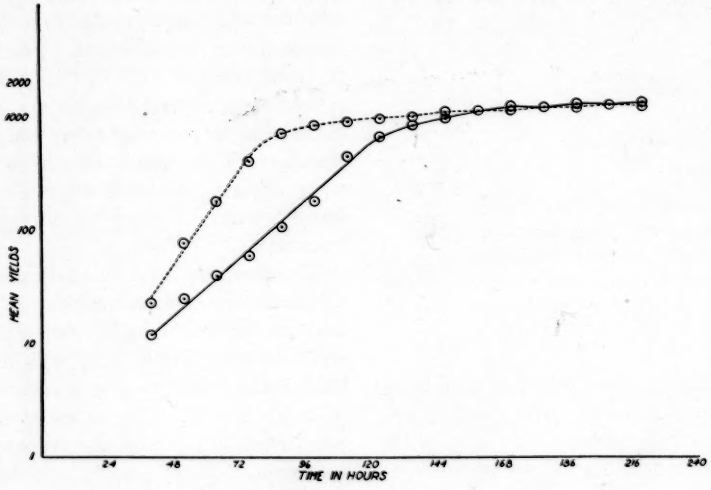

FIG. 1.—Comparison of growth rates of picric acid treated cultures with controls. Solid line indicates growth in control cultures. Interrupted line indicates growth in cultures containing 2×10^{-4} M picric acid.

DISCUSSION

It appears that picric acid concentrations of 1×10^{-4} M or 2×10^{-4} M have the effect of increasing the growth rate of *Tetrahymena* under all conditions tested, except where the growth rate is at a very high level. The only failure to stimulate occurred in cases where the medium had been autoclaved for a long period under acid or alkaline conditions and had presumably been hydrolyzed or altered in some way. In all these cases the growth in the hydrolyzed medium was unusually good, if the medium was allowed to remain acid or alkaline when used. If the lyzed as described, then readjusted to pH 7.0 before inoculation, gives a much stronger stimulation of growth by picric acid than medium which has not been hydrolyzed. This fact and the fact that increase in respiratory rate by picric acid was obtained only in the presence of the nutrient medium would seem to indicate that picric acid has something to do with the rate of penetration or utilization of the nutrient materials.

Within limits, increase in the amount of food has generally been found to increase the maximum population density of microörganisms. The fact that picric

acid causes no increase in the maximum density indicates that it probably is not being used as a food source. A further indication of this is that the concentration causing optimum stimulation is far too small to account in this way for such a sustained increase in growth rate as was found.

The growth-stimulating effect of picric acid was not so marked in aerated cultures as in nonaerated ones. From this it seems that the performance by picric acid of some functions usually performed by molecular oxygen in the medium may be the reason for at least part of the effect.

There seems to be very little similarity between the effects of picric acid and those of dinitrophenol. Under the conditions which gave a large picric acid stimulating effect on growth, dinitrophenol showed, at best, a very small effect. Further, the effect of picric acid on respiration was small.

SUMMARY

1. The growth rate of *T. geleii* is increased under certain conditions by picric acid concentrations of 1×10^{-4} M or 2×10^{-4} M, the optimum being about 2.2×10^{-4} M.

2. The most marked growth-stimulating effect of picric acid was obtained when using a slightly hydrolyzed proteose-peptone medium at pH 7.0. A growth-rate increase of as much as 60 per cent was obtained.

3. No increase in maximum population density is caused by picric acid.

4. It seems evident that the utilization of the picric acid as a food source is not the explanation of the increase in growth rate.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

THE OCEANS: THEIR PHYSICS, CHEMISTRY, AND GENERAL BIOLOGY[1] BY H. U. SVERDRUP, MARTIN W. JOHNSON, AND RICHARD H. FLEMING

Reviewed by GEORGE L. CLARKE

Harvard University and Woods Hole Oceanographic Institution

THE appearance of this volume represents the fulfilment of a long-felt need for an inclusive and critical treatment of modern oceanography. It comprises a thousand-page survey of well-established oceanographic knowledge and current theories in all the many branches of the subject—geographical, geological, physical, chemical, and biological. Nothing approaching the comprehensiveness of the present authoritative volume has appeared since *The Depths of the Ocean* (1912), by Murray and Hjort. Various scholarly works on individual subdivisions of oceanography have been published, it is true, and a few popular-styled short treatments of the whole subject have become available from time to time, such as Russell and Yonge's excellent book *The Seas* (1928 and 1936). Murray and Hjort's volume was based primarily on the investigations of one research vessel, and, although still valuable, it is now out of date because of the tremendous advances in oceanography during the last twenty-five years. Therefore, not only is *The Oceans* timely; it is also unique.

The authors of *The Oceans* state that "the close interrelation and mutual dependence of the single marine sciences have long been recognized" and that "the book is intended to provide a good deal of factual information, but above all it should be an aid to the beginner and specialist alike in the coordination of the various fields of oceanography." Their effort to attain these objectives is seen in the subjects of the chapters, for, although certain factual material is suitably segregated in chapters devoted to the physics, chemistry, geology, etc., of the sea, many more chapters are concerned with the influence of one branch of oceanography on another. This emphasis on interdependence appears especially in the biological parts of the work, in which several chapters are devoted to the ecological relationships of marine organisms to the physical and chemical influences of their environment. However, the reason for the particular sequence of chapters which has been adopted is not entirely clear; some further explanation of the arrangement of topics seems desirable for the orientation of the reader.

In the treatment of the text itself a happy combination has been attained in providing a wealth of information and a critical account of both practical investigations and theoretical studies. The authors state in their Preface that the discussion of many topics . . . . has led to tentative conclusions that are perhaps presented here

[1] New York: Prentice-Hall, Inc., 1942. Pp. x+1087+265 figs.+121 tables+7 charts. $8.00.

as better substantiated than is actually the case. At the risk of premature generalization we have, however, preferred definite statements to mere enumeration of uncorrelated observations and conflicting interpretations. . . . .

The valuable result of this policy has been that the more stimulating current problems in oceanography have been brought into relief against a background of the essential factual material. The authors have added their own appraisal, digestion, and interpretation of the investigations which are reviewed. This characteristic more than any other makes the book the outstanding contribution that it is.

After a brief historical sketch of the development of oceanography, successive chapters of *The Oceans* deal with the topography of the ocean basins, the physical properties of sea water, the facts and theories of the distribution of variables, the chemistry of sea water and the composition of marine organisms, the sea as a biological environment, and an enumeration of the types of plants and animals that live in it. Another group of chapters is concerned with methods of making observations and collections at sea; the general character and dynamics of ocean currents, waves, and tides; and marine sedimentation.

The population physiologist and the ecologist will be especially concerned with a group of four chapters comprising a readable and authoritative discussion of the relations of the phytoplankton and of animals to the physical-chemical properties of the environment, the interrelations of marine organisms, and organic production in the sea. Here is assembled a comprehensive account, with illustrations, of the facts and theories of marine ecology. The influence of such factors as light, temperature, nutrients, and oceanic circulation in controlling the growth, distribution, and population density of plants and animals in the sea is discussed in detail. The significance of size, numbers, feeding methods, and nutritional requirements is emphasized, particularly in relation to the fundamental cycle of life in the sea. Considerable space is given to the role of bacteria in the biological and chemical cycles of the free water and of the ocean bottom. Finally, the production of phytoplankton, zoöplankton, and "commercial" organisms is appraised and the production of different regions compared.

In general, the appearance and physical arrangement of the volume are excellent. Since the book embraces over one thousand pages of text, an appendix of tables, and seven folded charts, it is necessarily rather bulky, and some would perhaps find it more serviceable if it were bound in two volumes. On the other hand, the careful subdivision into twenty chapters of the vast amount of material covered and the many headings and subheadings which appear in each chapter, in addition to the "Subject Index" and the "Index of Names" for the whole book, facilitate the ready location by the reader of any desired topic. An extensive bibliography is provided at the end of each chapter. The book is replete with excellent diagrams and tables, many of them original and many arranged to summarize or to compare information derived from a variety of sources. The treatment of the book as a whole is therefore a very successful realization of the authors' aim—to provide in *The Oceans* a summary of the established facts of oceanography and a synthesis and evaluation of current theories.

BIOCHEMISTRY AND MORPHOGENESIS[1] BY JOSEPH NEEDHAM

Reviewed by J. H. BODINE
State University of Iowa

THIS book is in no way a mere repetition of the author's former one, *Chemical Embryology*, since its subject matter deals almost solely with "the biochemical investigation of what may truly be called the morphogenetic hormones." The book is divided into three parts: (I) the morphogenetic substratum, (II) the morphogenetic stimuli, and (III) the morphogenetic mechanisms.

Part I treats of the chemical matter of which the egg and embryo are built. Part II, making up the greater part of the book, deals with the organizers, inductors, genes, and other chemical and physicochemical factors that have to do with the initiation and patterns of development in organisms. Part III, dealing with mechanisms, emphasizes such phenomena as growth, differentiation, respiration, and metabolism of the developing embryo.

Any attempt to give an adequate concept of the detailed contents of the book is quite impossible because of its encyclopedic nature. Without question, the subject matter is intensively and exhaustively discussed without undue complication or repetition. Data are given for all animals, with adequate and accurate author references. The subject matter is orderly and logically arranged, with no attempt on the part of the author other than to present complete pictures of all available information on the various topics discussed. A thoroughgoing and complete grasp of the subject matter by the author is at all times apparent. Plates, graphs, and other illustrative materials are well chosen and are generously supplied throughout. In many cases original graphs of what would otherwise be confusing data are presented in the author's usual lucid fashion. Adequate reference is at all times made to original figures shown. Reproductions of photographs are especially clear and numerous.

The book in scope of references and data is truly cosmopolitan and represents no single school of thought. It contains a glossary, a well-indexed and keyed Bibliography, a general index as well as an index of animals, plants, and genes. All of these are complete and leave little to be desired in making the book of especial value to the experimentalist, be he chemist or biologist.

Clarity and simplicity of style, along with accurate analysis and reproduction of data, should make this book of especial value to those wishing a readily available source of information on so vast a field as biochemistry and morphogenesis.

[1] New York: Cambridge University Press, 1942. Pp. x+787+328 figs. $12.50.